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(54) Title: HERBICIDES TEST METHOD

(57) Abstract

An isolated DNA molecule encoding a protein from a plant, which protein has pantothenate synthetase activity; a non–naturally occuring chimeric gene comprising a promoter operably linked to a DNA molecule encoding a protein from a plant having pantothenate synthetase activity; a recombinant vector comprising the chimeric gene wherein the vector is capable of being stably transformed into host cell, a host cell stably transformed with a vector wherein the host cell is capable of expressing the DNA molecule; a method for assaying a protein having pantothenate synthetase activity; the use as herbicides of compounds which inhibit pantothenate synthetase, and a herbicidal composition, comprising one or more active ingredients which show significant pantothenate synthetase inhibition in an assay, are disclosed.

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HERBICIDES TEST METHOD

The invention relates to plant enzymatic activity, and aspects thereof, involved in the biosynthesis of Coenzyme A. The invention particularly relates to the plant enzyme known either as pantoate-β-alanine ligase (EC 6.3.2.1), pantoate activating enzyme or pantothenate synthetase (PS). PS catalyses the synthesis of pantothenate.

PS is an essential enzyme in the *in planta* biosynthesis of the vitamin and Coenzyme A precursor pantothenate. It is known to catalyse the following reaction:

ATP + (*R*)-pantoate + β-alanine → AMP + pyrophosphate + (*R*)-pantothenate.

PS genes have previously been isolated from *Escherichia coli* (GenBank accession number P31663), *Bacillus subtilis* (GenBank accession number P52998), and the cyanobacterium *Synechocystis* (GenBank accession number U44896). DNA sequences from *Saccharomyces cerevisiae* (GenBank accession number P40459) and *Schizosaccharomyces pombe* (GenBank accession number Q09673) having unknown functions have been proposed to code for PS enzymes based on DNA and deduced amino acid sequence similarities. To date, however, no gene has been reported which codes for the PS enzyme in any plant species. It is therefore an object of the invention to identify, isolate and sequence a gene coding for the PS enzyme present in plants.

A number of assays have been reported for measuring PS activity. One assay developed by Maas (1950a and 1950b) uses a microbiological assay of pantothenate based on the ability to promote growth of an *E. coli* pantothenate auxotroph (M99-1, *panC*). The assay developed by Pfleiderer *et al* (1960) measures the AMP liberated in the PS reaction. In this assay, myokinase catalyses the production of 2 moles of ADP for each mole of AMP released in

pantothenate synth sis using ATP supplied in the assay mixture. Pyruvat kinase then generates 2 moles of pyruvat and ATP for 2 moles of phospho nolpyruvat and ADP. Finally, lactate dehydrogenase reduces 2 moles of pyruvate to yield 2 moles of lactate concomitant with stoichiom tric oxidation of NADH to NAD, which can be monitored spectrophotometrically by following the absorbance at 340 nm. A third assay, developed by Miyatake *et al* (1979), employs an assay mix containing ^{14}C-β-alanine and unlabelled pantoate. In this assay any ^{14}C-pantothenate formed is separated from unreacted ^{14}C-β-alanine by cation exchange chromatography and subsequently quantified by liquid scintillation counting. These assays, however, are not suitable for use with high throughput biochemical screening and cannot be used for the large scale biochemical screening of compounds necessary to discover useful inhibitors of PS.

We have developed an invention which addresses the above-mentioned drawbacks associated with the prior art. Our invention covers a number of related aspects which encompass the same inventive concept.

According to a first aspect of the invention there is provided an isolated DNA molecule encoding a protein from a plant, which protein has PS activity. In preferred embodiments, the DNA is isolated from *Lotus japonicus* or *Oryza sativa.*

To support our invention we herein disclose the cDNA sequence from *Lotus japonicus*. In addition, we have shown that a previously unassigned expressed sequence tag of *Oryza sativa* (GenBank accession number D25017) is part of a cDNA coding sequence for a PS enzyme in *Oryza sativa* and disclose, as part of this invention, the full cDNA sequence of the PS gene from *Oryza sativa*. Furthermore, we have confirmed by sequence similarity, functional complementation of an *Escherichia coli* mutant devoid of PS enzyme activity, and by enzyme assays that the DNA s quence from *Saccharomyces cerevisiae* (GenBank accession number P40459) putatively ascribed as coding

for a PS enzyme does code for the PS enzyme of *Saccharomyces cerevisia* . A cDNA sequence coding for a PS enzyme in *Lotus japonicus* is provided in Figure 1.2. A cDNA sequence coding for a PS enzyme in *Oryza sativa* is provided in Figure 2.2. A DNA sequence coding for a PS enzyme in *Saccharomyces cerevisiae* is provided in Figure 3.3. As a result of our invention it is now possible to obtain the DNA coding sequence for the PS enzyme(s) from any plant source using methods available to those skilled in the art.

A further preferred embodiment of this aspect of our invention is an isolated DNA molecule encoding a protein from *Lotus japonicus* having PS activity wherein said protein comprises the amino acid sequence set forth in Figure 1.2. A still further embodiment is an isolated DNA molecule encoding a protein from *Oryza sativa* having PS activity wherein said protein comprises the amino acid sequence in Figure 2.2.

In addition, we have extended our invention to include a further aspect so as to provide a non-naturally occurring chimeric gene comprising a promoter operably linked to a DNA molecule encoding a protein from a plant having PS activity. Preferably, the protein is isolated from a dicotyledonous or a monocotyledonous plant, such as *Lotus japonicus* or *Oryza sativa*. Preferably the amino acid sequence is selected from the group set forth in Figure 1.2 (*Lotus japonicus*) and Figure 2.2 (*Oryza sativa)*.

We have developed our invention into another aspect which provides a recombinant vector comprising a chimeric gene, wherein the vector is capable of being stably transformed into a host cell. Also comprised in this aspect is the host cell stably transformed with the vector wherein the host cell is preferably a cell selected from the group consisting of a bacterial cell, a yeast cell, and an insect cell and is further capable of expressing the DNA molecule according to the invention.

In a still furth r aspect we have applied our invention to the recombinant production of the PS enzyme. In particular, the invention provid s a method of producing a prot in having PS activity in a host organism by firstly inserting a DNA sequence encoding a protein having PS activity into an expression cassette designed for the chosen host; inserting the resultant molecule, containing the individual elements linked in proper reading frame, into a vector capable of being transformed into the host cell; growing the thus transformed host cell in a suitable culture medium; and isolating the protein product either from the transformed cell or the culture medium, or both, and purifying it.

In addition, we have developed our invention to provide methods for assaying a protein having pantothenate synthetase activity comprising; incubating pantothenate synthetase in a suitable reaction mixture in which pantothenate synthetase is capable of catalysing the conversion of pantoate, □-alanine and ATP to pantothenate, AMP and pyrophosphate; determining the amount of pyrophosphate formed by a colorimetric technique based on the assay for pyrophosphate developed by Chang *et al.* (1983); or converting the pyrophosphate formed by the catalytic activity of the pantothenate synthetase into inorganic phosphate by the catalytic activity of an inorganic pyrophosphatase, preferably yeast inorganic pyrophosphatase; and determining the amount of inorganic phosphate generated by the catalytic activity of said inorganic pyrophosphatase by colorimetric techniques, preferably by techniques based either on the assay for inorganic phosphate developed by Lanzetta *et al.* (1979) or on the assay for inorganic phosphate developed by Chifflet *et al.* (1988).

The production of PS, for example by using the recombinant methodology described hereinabove, has enabled us to develop methods of using purified PS to screen for novel inhibitors of PS activity which may be used as herbicides to control undesirable vegetation in fields where crops are grown, particularly agronomically important crops such as maiz and other cereal crops such as wheat, oats, rye, sorghum, rice, barley, millet, turf and forage

grasses, and the like, as well as cotton sugar cane, sugar beet, oilse d rape, and soybeans.

In particular, the present invention relates to a method for assaying a chemical entity for the ability to inhibit the activity of a PS enzyme from a plant by:

a) combining said PS enzyme in a suitable reaction mixture in which

i) said PS enzyme is capable of catalysing the conversion of pantoate, ß-alanine and ATP to pantothenate, AMP and pyrophosphate;

ii) the pyrophosphate liberated in the PS reaction is determined by a colorimetric method, preferably by a method based on the assay for pyrophosphate developed by Chang *et al.* (1983);

iii) or the pyrophosphate liberated in the PS reaction is further converted to inorganic phosphate by the catalytic activity of an inorganic pyrophosphatase, preferably yeast inorganic pyrophosphatase; and

iv) the inorganic phosphate generated by the catalytic activity of inorganic pyrophosphatase is determined by a colorimetric method, preferably by a method based on the assay for inorganic phosphate developed by Lanzetta *et al.* (1979) or by a method based on the assay for inorganic phosphate developed by Chifflet *et al.* (1983);

b) combining said chemical and said PS enzyme together in a second reaction mixture under the same conditions as in said first reaction mixture; and

c) measuring the amount of pyrophosphate or inorganic phosphate produced in said first and said - second reaction mixture;

wherein said chemical is capable of inhibiting the activity of said PS enzyme if the amount of pyrophosphate or inorganic phosphate measured in said second reaction mixture is significantly less than the amount of pyrophosphate or inorganic phosphate measured in said first reaction mixture.

Th assay principle invented here is not limited to measuring PS activity but can be mploy d to measure any enzyme whose catalytic activity involv s the formation of a substrate-nucleotidyl reaction intermediat by transfer of the nucleotidyl moiety from the corresponding nucleoside triphosphate to a suitable substrate, thereby generating inorganic pyrophosphate as one reaction product. Such enzymes include, but are not limited to, all aminoacyl-tRNA synthetases, asparagine synthetase, acetate thiokinase, dephosphocoenzyme-A-pyrophosphorylase and all enzymes catalysing the formation of nucleotide-diphosphate-sugars. The assays are preferably carried out on a microtiter scale and are preferably employed for the high-throughput biochemical screening of inhibitors of the enzymes.

The present invention is further directed to probes capable of specifically hybridising to a plant PS gene, cDNA or mRNA, wherein the probe comprises a contiguous portion of the coding sequence for a PS enzyme from a plant at least 10 nucleotides in length.

A further aspect the invention provides a method of producing a DNA molecule comprising a DNA portion encoding a protein having PS activity by,

a) preparing a nucleotide probe capable of specifically hybridising to a plant PS gene, cDNA or mRNA, wherein the probe comprises a contiguous portion of the coding sequence for a PS enzyme from a plant at least 10 nucleotides in length;
b) probing for other PS coding sequences in populations of cloned genomic DNA fragments or cDNA fragments from a chosen organism using the nucleotide probe prepared according to step a); and
c) isolating a DNA molecule comprising a DNA portion encoding a protein having PS activity.

DNA encoding the PS enzyme may be isolated from any desired plant species according to the invention. One method taught for isolating a plant PS coding sequence is represented by Example 1. In this method cDNA clon s encoding a PS enzyme are identified from a library of cDNA clones derived from the

plant of interest based on their ability to supply PS enzymatic activity to a mutant host organism deficient in this activity. Suitable host organisms for us in this m thod are those which can be used to screen cDNA expression libraries and for which mutants deficient in PS activity are either available or can be routinely generated. Such host organisms include, but are not limited to, *E. coli* panC (strain AT1371).

Alternatively, plant PS coding sequences may be isolated according to well known techniques based on their sequence homology to the *Lotus japonicus* PS coding sequence set forth in Figure 1.2 or to the *Oryza sativa* PS coding sequence set forth in Figure 2.2. In these techniques all or part of the known PS coding sequence is used as a probe which selectively hybridises to other PS coding sequences present in populations of cloned genomic DNA fragments or cDNA fragments (i.e. genomic or cDNA libraries) from a chosen organism. Such state of the art techniques include hybridisation screening of plated DNA libraries and amplification by PCR using oligonucleotide primers corresponding to sequences conserved among known PS amino acid sequences.

For recombinant production of the PS enzyme in a host organism, the plant PS coding sequence may be inserted into an expression cassette designed for the chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5´and 3´untranslated sequences, and enhancer appropriate for the chosen host is within the level of skill of those skilled in the art. The resultant molecule, containing the individual elements linked in proper reading frame, may be inserted into a vector capable of being transformed into the host cell. Suitable expression vectors and methods for recombinant production of proteins are well known for host organisms such as *E. coli*, yeast and insect cells. Specific examples include plasmids such as pBLUESCRIPT, pFLAG, pTrcHis, and baculovirus expression vectors, for example those derived from the genome of *Autographica californica* nuclear polyh drus virus.

Recombinantly produced plant PS can be isolated and purified using a variety of standard techniques. The actual techniques which may be used will vary depending upon the host organism used, whether the PS enzyme is designed for secretion, and other such factors familiar to those skilled in the art.

Recombinantly produced plant PS is useful for a variety of purposes. For example, it may be used in an *in vitro* assay to screen known herbicidal chemicals whose target has not been identified to determine if they inhibit PS. Such an *in vitro* assay may also be used as a more general screen to identify chemicals which inhibit PS activity and which are therefore herbicide candidates. Alternatively, recombinantly produced plant PS may be used to elucidate the complex structure of this enzyme. Such information regarding the structure of the PS enzyme may be used, for example, in the rational design of new inhibitory herbicides.
Typically, the inhibitory effect on PS is determined by a significant reduction, a reduction that is greater than the margin of error inherent in the measurement technique, of pantothenate synthesis in the *in vitro* assay. Such a determination may be made simply by comparing the amount of pantothenate synthesised in the *in vitro* assay in the presence and absence of the candidate inhibitor.

The disclosures in British patent applications 97 111 63.7 and 97 134 77.9 from which this application claims priority, and in the abstract accompanying this application are incorporated herein by reference.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Examples

A number of standard techniques have been used during development of our invention. These includes: cloning of plant genes by functional complementation (for example Senecoff and Meagher, 1993); the use of inverse PCR to recover fragments of genes not present in conventional

libraries (Ocham *et al*, 1989) and the use of DNA sequence databases to discover PS genes cloned from other species which had unknown function at the time of their submission.

Example 1

Isolation of a cDNA clone encoding pantothenate synthetase from *Lotus japonicus*

A *L. japonicus* PS clone was isolated by functional complementation of *E. coli* AT1371 (*panC4, Δ(gpt-proA)62, lacY1, tsx-29, glnV44(AS), galK2, λ⁻, rac-0, hisG4(Oc), rfbD1, xylA5, mtl-1, argE3(Oc), thi-1*, described by Cronan *et al.*, 1982) from a cDNA library (from Corinna Tetzlaff, Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK.) The PS cDNA was found in a population of 50,000 ampicillin-resistant transformants of *E. coli* AT1371. The PS clone (pLC), was subcloned and sequenced as summarised in Figure 1.1. The resulting nucleotide sequence (Figure 1.2) revealed the presence of an open reading frame (ORF) encoding a polypeptide of 308 residues which is 61% similar to the protein sequence of PS from *E. coli*. The open reading frame of PS was in frame with the *lacZ* on the pBLUESCRIPT vector which probably accounts for the expression of *L. japonicus* PS and hence complementation of E. coli AT1371.

Example 2

Isolation of a cDNA clone encoding pantothenate synthetase from *Oryza sativa*

A PS cDNA sequence from rice was found by nucleotide database searches as an expressed sequence tag (EST) of rice that had been submitted to GenBank (accession number D25017) on behalf of the Japanese Rice Genome Research Program. The full corresponding cDNA clone was obtained from Dr. Yuzo Minobe, National Institute of Agrobiological Resources, Kannondai, Tsukuba Ibaraki, Japan. This cDNA clone was called pRC1 and subcloned and sequenced as shown in Figure 2.1. The nucleotide sequence of the 1.3kb *SalI-NotI* insert of pRC1 and the predicted amino acid sequence of the PS gene are given in Figure 2.2. When this sequence was compared to other PS sequences, the similarity originally seen within the 5' EST region held for the

entire op n reading frame implying that the rice cDNA in pRC1 does code for PS. However, pRC1 did not complement the *E. coli panC* mutant. Thus, a fusion clone of *lacZ* and rice PS gene was derived from pRC2 (Figure 2.3) that allowed both transcription of the rice cDNA and translation of the protein as a β-galactosidase fusion. Complementation of the *E. coli panC* mutant with the rice pantothenate synthase gene was achieved using this fusion clone.

Example 3

Comparison of the amino acid sequences of known, or predicted to be, pantothenate sythetases

When the amino acid sequences of known, or predicted to be, pantothenate sythetases were aligned (Figure 3.1), the putative PS protein sequences of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* showed significant homology with *E. coli* PS. To confirm that the putative PS gene of *Saccharomyces cerevisiae* did code for an enzyme with PS activity, a phage clone (PM4950) containing a 20 kb genomic fragment of the yeast chromosome IX which spans the putative PS gene was obtained from Dr. Carol Churcher, Sanger Centre, Hinxton Hall, Cambridge, UK. The open reading frame coding for the putative gene was subcloned in two steps for expression in *E. coli* as shown in Figure 3.2. In the resulting plasmid, pYC1, the yeast PS gene was under transcriptional control of the *lacZ* promoter. However, PS was not in frame with *lacZ* and therefore was not expressed as a fusion protein. The nucleotide sequence and putative translation product of the 1.5 kb *Eco*RV-*Hind*III genomic fragment in pYC1 are given in Figure 3.3. Yeast PS functionally complemented the *panC* lesion in *E. coli* AT1371, confirming that the gene did code for a functional PS.

Example 4

Isolation of the 5' and the 3' ends of pantothenate synthetase from *Lotus japonicus*

We expected plant PS to be located in the chloroplast, but there was no evidence from the PS cDNA clones of *L. japonicus* and *O. sativa* of any

chloroplast transit signals. Furthermore, both enzymes were predict d to be cytosolic proteins by PSORT (Molecular Biology Tools, ExPasy WWW S rver).

To clone 5' and 3' sequences of the PS gene inverse PCR (iPCR) was used. Genomic DNA was isolated from *L. japonicus* leaf tissue and prepared as described by Dellaporta *et al* (1983). Aliquots of *Lotus* genomic DNA (8 µg) were digested overnight with the following restriction endonucleases: *Bam*HI, *Eco*RI, *Hind*III, *Not*I, *Sal*I, *Xba*I, *Xho*I. DNA fragments were precipitated with isopropanol and resuspended in TE buffer before loading onto an agarose gel. After electrophoresis, gel pieces corresponding to fragment sizes of between 2 kb and 15 kb were isolated from the agarose gel, and purified using the US BioClean MP kit (United States Biochemical, Cleveland, Ohio, USA). Each reaction was ligated overnight at 14°C with 1.5 units of T4 DNA ligase in conditions which promote intra-molecular ligation. DNA was precipitated from the ligation mix with isopropanol, washed with ethanol, resuspended in sterile distilled water and used as templates in the following PCR step.

PCR amplification was carried out using the Expand High Fidelity PCR system from Boehringer Mannheim, FRG, adopting the manufacturers protocol for amplification of DNA of a size of up to 3 kb. The design of *L. japonicus* PS-specific primers Li5 and Li3 (**Li5**: dCGGGATCCATGGTGGGAACGAGGGCGATGAG and **Li3**: dCATCAAGCTTATGTATCAAAGTGCCCCAGG) followed the general protocols for iPCR by Ochman *et al.* (1989). Restriction sites incorporated into the Li primers are *Hind*III in Li3 and *Bam*HI in Li5 (underlined). Of all seven templates only the *Bam*HI-derived circular *Lotus* library was effective in iPCR reactions. A single product of 750 bp was obtained with a minor contaminant at about 250bp. This iPCR product was cleaved by *Bam*HI as expected and was cloned into the pCRII vector using the PCR T/A cloning kit (Invitrogen, NV Leek, Netherlands following the manufacturer's instructions). The *Eco*RI insert of the resulting plasmid was sequenced at the Centre of Molecular Recognition, Department of Biochemistry, University of Cambridge (Figure 4 A). By comparison with the *L. japonicus* PS sequence, the genomic iPCR product

matched the cDNA's 3' and 5' ends and was assumed to contain authentic PS flanking r gions. Th annotated nucleotide s quence is shown in Figure 4 B.

While primer and cDNA sequences were straightforwardly identified within the iPCR product, analysis of the flanking regions was more difficult, since intron-exon borders can only be identified with some confidence. Prediction of splice sites according to Hebsgaard et al. (1996) at the NetPlantGene Server (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark), suggested possible donor splice sites at positions 258 and 281, and possible acceptor splice sites at positions 179, 316, 338 and 536 in the sequence given in Figure 4B. These sites were found in the low probability threshold mode of the program to include nearly all true sites. Intriguingly, none of these splice sites are situated within regions corresponding to cDNA.

There is one sequence feature of value regarding the question of the true translation start site within PS. This is a stop codon in the 5' flanking region, 21 bases upstream from the putative initiation site, which is in frame with the PS ORF. If this stop codon forms part of the PS 5'-leader sequence as was implied in the splice site predictions, the ORF as encoded by the PS cDNA could safely be assumed to be complete. Specifically, this conclusion is conditional upon the absence of acceptor splice sites in between stop codon and cDNA start. The primary sequence information as such seems reliable firstly because a DNA-polymerase with proof reading activity was used in the PCR amplification and secondly because the chromatograms generated in two sequencing runs are both unambiguous.

Example 5

Cloning pantothenate synthetase from *Lotus japonicus* into an expression vector

An expression cassette was generated from the *L. japonicus* cDNA for PS using the PCR method of MacFerrin *et al* (1990). *Lotus pan*C ORF was amplified from *ca*. 25 ng of plasmid pLC, using the primers LC5 and LC3. LC5 was designed to the start, ATG, codon of PS with an *Xba*I site highlighted in

bold type and a ribsome binding site, underlined, included in th PCR primer:

dCGCGC**TCTAGA**AGGAGGAATTTAAA**ATG**GCACCAATGGTGATATCTGAT.

LC3 was designed to include the stop codon (TTA) of the ORF and an *Xho*I restriction site, in bold, in the PCR primer:

dGCGCGC**TCGAG**TTACAAGTTGATTTCTATGTT.

The PCR product was cloned into pBLUESCRIPT SK⁻ (Stratagene Ltd. Cambridge Innovation Centre, 140 Cambridge Science Park, Milton Road, Cambridge, CB4 4GF, UK) using the *Xba*I and *Xho*I restriction sites incorporated in the primers, and the resulting clone was referred to as pSKL. The expression cassette was designed to contain the *L. japonicus* PS ORF as demonstrated Figure 5. The correct construct was confirmed by DNA sequence analysis (data not shown).

Example 6

Expression of pantothenate synthetases from *Lotus japonicus, Oryza sativa,* and *Saccharomyces cerevisiae* in *Escherichia coli*

L. japonicus PS was expressed in an *E. coli panC* mutant AT1371 because wild-type *E. coli* strains have considerable PS activity which would make purification of the recombinant enzyme more difficult. *E. coli* AT1371 (*panC*) transformed with the *Lotus panC* overexpressing plasmid pSKL was grown from single colonies overnight in 10 ml LB cultures containing 100 µg/ml ampicillin. Four 500 ml aliquots of 2YT medium (1.6% (w/v) bactopeptone, 1.0% (w/v) yeast extract and 0.5% (w/v) NaCl in water) in 2 litre flasks containing 60 µg/ml ampicillin and 20 µg/ml IPTG were each inoculated with 5 ml of overnight culture and incubated at 37°C with shaking (190 RPM) for 10 hours before harvesting. *E. coli* cells were recovered by centrifugation (10 min, 5000 RPM) and immediately resuspended in 20 ml of buffer A (50 mM Tris·HCl, 1 mM EDTA, 0.1 mM DTT, pH 8.0). Cells were lysed by sonication. Two equal aliquots of the cell suspension were each sonicated 6 times for 30 seconds on ice, with a 30 second pause between each burst. Cell debris was removed by

centrifugation (30 min, 12000 RPM) and the crude extract was assayed for enzymatic activity.

Along with the *Lotus* PS expression clone pSKL, expression of PS activity was examined with all other available PS clones, that is the *lacZ*-PS fusion clones of *Lotus* and rice (pLC and pRC, respectively), yeast *panC* (pYC1) and *E. coli panC* (pCL). However, unlike pSKL clones, no attempt was made to optimise expression. Crude extracts from *E. coli* AT1371 transformed with these pantothenate synthase clones or with vector alone were assayed for pantothenate synthase activity using either pantoate or pantoyl-lactone as substrate. Crude extract from wild type *E. coli* was also assayed, and the results are shown in the appended table (Table 1) which includes previously reported PS activities. In all cases examined enzyme activities were much higher with pantoate than with pantoyl-lactone. Given that purified *E. coli* PS had no activity toward pantoyl-lactone (Miyatake *et al.*, 1979), the residual activities seen here with the latter substrate are likely due to a hydrolysing activity present in the cell extracts. An activity that catalyses hydrolysis of pantoyl-lactone was previously implied by Maas (1952a and 1952b). Failure to detect activity in samples derived from the *Lotus* or rice *panC-lacZ* fusion clones indicates lack of expression of enzymatically active PS. However, these clones were successfully used to complement a *panC* lesion in *E. coli* and therefore must express at least low levels of PS activity. Activities found in wild type *E. coli* or AT1371 transformed with vector alone are in accordance with previously reported values.

Example 7

Purification of recombinant *Lotus japonicus* pantothenate synthetase expressed in *Escherichia coli*

A crude extract of *E. coli* AT1371 transformed with the *Lotus panC* expression clone pSKL was prepared as described in Example 6. Starting from this crude extract, PS was essentially purified in two steps, ammonium sulphate fractionation and anion exchange chromatography. To the cleared extract (28 ml), a saturated solution of $(NH_4)_2SO_4$ (12 ml) was added to reach a final

concentration of 30% $(NH_4)_2SO_4$. The solution was kept on ice for 1 hour with stirring to allow protein aggregates to form. Insoluble protein was removed by centrifugation (12,000 RPM, 30 minutes) at 4°C. The supernatant was recovered (36 ml) and brought to 40% $(NH_4)_2SO_4$ saturation by addition of 6 ml of saturated $(NH_4)_2SO_4$ solution. The solution was incubated and centrifuged as before. Pelleted protein aggregates were dissolved in 5 ml of buffer A and dialysed against 2 litres of buffer A overnight at 4 °C. The dialysed solution was centrifuged at 12,000 RPM for 30 minutes, and the supernatant was directly used in the anion exchange chromatography step.

The sample (5 ml) was loaded onto a Pharmacia FPLC MonoQ HR10/10 column previously equilibrated in buffer A. The column was washed with equilibration buffer until A_{280} of the eluate was constant and below 0.1, and a constant flow rate of 2 ml/min was maintained throughout the run. Protein was eluted in a linear gradient (80 ml) of 0 - 250 mM KCl in buffer A, and 1 ml fractions were collected throughout the gradient and assayed for PS activity. Figure 6 shows the PS activity (Figure 6A) and protein (Figure 6B) profiles of this chromatographic step. The majority of PS activity eluted at *ca*. 100 mM KCl concentration. A second peak of PS activity eluted well separated from the first at just under 200 mM KCl along the gradient. This peak was broader than the first one and contained a much smaller but significant amount of activity. Since a homogenous overexpression product would principally be expected to elute in a single peak, separation of PS activity into two peaks was at first held to be an artifact. However, using a different column (MonoQ HR16/10) or changing gradient parameters including a solute change from KCl to ammonium acetate made no difference to the elution pattern. Physical differences in between the PS proteins in peaks one and two that could account for this behaviour may be due to differential folding or post-translational processing. The fractions with highest PS activities in either peak were pooled as indicated in Figure 6A. Fractions 29 through 32 within the first peak gave sample PS-I (4 ml) which recovered 73% of PS activity loaded onto the column. Likewise, fractions 57 through 60 from the second peak were pooled to give PS-II (4 ml) containing

12% of the original PS activity. Samples PS-I and PS-II together contained 21.7 mg of PS.

Both, PS-I and PS-II were dialysed against 1 litre of buffer A overnight at 4 °C and centrifuged to precipitate insoluble protein. 500 µl aliquots of both PS-I and PS-II were loaded onto a Pharmacia Superose 6 column equilibrated in buffer A, maintaining a constant flow rate of 0.5 ml per minute and collecting 1 ml fractions. PS activity from both samples eluted in single peaks at an equal retention volume after injection, indicating a similar native molecular weight for PS-I and PS-II. This step offers no further purification of PS. In fact, specific activity was slightly decreased in both cases as can be seen from the purification summary in Table 2. However, SDS-PAGE (Laemmli, 1970; Sambrook *et al.*, 1989) analysis of these samples revealed removal of some protein contaminants through gel filtration. Fractions 16 and 17 were pooled in each case to give samples PS-I/GF and PS-II/GF. Physical characterisation of recombinant PS is dealt with in the next section and was carried out on both PS-I and PS-II while kinetic analysis (Example 9) was restricted to PS-I.

Example 8

Characterisation of the recombinant *Lotus japonicus* pantothenate synthetase

In order to confirm the identity of the overexpressed *Lotus* PS, N-terminal protein sequencing (Table 3) and amino acid analysis (Table 4) was carried out at the Protein and Nucleic acid Chemistry Facility in the Department of Biochemistry, University of Cambridge on an applied Biosystems 477A Protein Sequencer for both PS-I and PS-II.

Alignment of N-terminal sequences obtained for PS-I and PS-II to the theoretical N-terminus of *L. japonicus* PS in Table 3 demonstrated that the purified recombinant protein is PS from *L. japonicus*. The overexpressed protein was apparently processed at the N-terminus, and the majority of both PS-I and PS-II lacked two N-terminal residues (methionine and alanine). However, some of the protein only lacked methionine giving rise to the secondary sequences observed. PS-I and PS-II differ somewhat with respect to

the proportions of these differentially processed species. This can be s en from the relative yields at which primary and secondary sequences w re obtained. Th less abundant protein species with N-terminal alanine sequenced at an average yield comprising 70% (PS-I) or 40% (PS-II) of that seen for the primary sequence.

The theoretical molecular weight of recombinant *L. japonicus* PS is 34.2 kD, and this value is in reasonable agreement with the subunit weight obtained by SDS-PAGE analysis of the purified overexpression product (ca. 37 kD). The native molecular weight of PS-I or PS-II was estimated by gel filtration to be 72.8 kD implying the native protein is a homodimer.

More accurate determination of the *Lotus* PS subunit molecular weight was achieved by electrospray mass spectroscopy ESMS (carried out on an electrospray ionisation (positive ion mode) quadrapole mass spectrometer (BioQ; VG, Manchester, UK) using software supplied by the manufacturer). The transformed mass data revealed the presence of two protein species both in PS-I and PS-II which differ by 72.3 Da and 70.3 Da, respectively. This corresponds well to the theoretically expected mass difference in between presence or absence of an N-terminal alanine, that is 71.0 Da. Protein sequencing of recombinant PS had already shown that the N-terminal methionine was missing from the overexpression product while the following alanine residue was only partially removed. The main ESMS signal (100%) belongs to the lighter species and does therefore in all likelihood correspond to PS with N-terminal proline. Likewise, the secondary signals obtained at 75% (PS-I) or 40% (PS-II) relative intensity are due to PS with N-terminal alanine. As was concluded from the N-terminal sequencing data, the relative proportions of lighter and heavier PS species obtained here indicate PS-II was more efficiently processed than PS-I.

The *L. japonicus* PS ORF encodes a polypeptide of 308 residues with a predict d molecular weight of 34.2 kDa, and the processed recombinant protein (3-proline through 308-leucine) has a theoretical mass of 34,037.7 Da.

This is only in rough accordance with the weights obtained for PS-I (33,969.0 ± 12.3 Da) and PS-II (33,967.0 ± 10.2 Da), that is these proteins are lighter than xpected by 68.7 Da and 70.7 Da, respectively. Given th accuracy of ESMS mass determinations, this discrepancy is presumably not due to a machine artifact. A possible explanation for the mass differences would be a mutation in the overexpression clone that might have been introduced through the PCR amplification of the *L. japonicus panC* expression cassette. However, the PCR step in question was carried out using a polymerase mix including a proof-reading activity, and, as mentioned earlier, no nucleotide sequence changes were found in between *panC* cDNA and expression cassette. Alternatively, the overexpressed PS may have been further processed at the C-terminus, for example.

Example 9

A high-throughput assay for pantothenate synthetase activity

Three different assays have been reported previously for measuring PS activity (see above). The assays described by Maas (1950a and 1950b) and Miyatake *et al* (1979) are either microbiological or radiometric, while the number of auxilary enzymes and substrates required for the assay developed by Pfleiderer *et al* (1960) makes this assay cumbersome, expensive, and limited in its application to low throughput screening only. Hence, all three assays are unsuitable for the large scale high throughput biochemical screening of compounds necessary to discover new inhibitors of PS.

The applicants have developed *in vitro* assays which can be employed for high throughput biochemical screening for detecting inhibitors of this enzyme, to the use of these assays in the development of novel herbicides and in determining their mode of action, and to biological active inhibitors of pantothenate biosynthesis and herbicides obtained thereby. The assays are designed to measure the pyrophosphate liberated in the PS reaction either directly with a modified version of the colorimetric assay for the determination of inorganic pyrophosphate originally described by Chang *et al.* (1983); or aft r its conversion with inorganic pyrophosphatase to inorganic phosphate, which is

then d termined with modified versions of the colorimetric assays for the determination of inorganic phosphate originally described by Lanz tta *et al.* (1979) or Chifflet *et al.* (1988). The assays are carried out at room temperature, preferably on a microtiter scale.

1. The preferred assay mixture to colorimetrically measure the pyrophosphate liberated in the PS reaction comprises 100 µmol Tris·HCl (pH 8.0), 10 µmol $MgSO_4$, 5 µmol ATP, 10 µmol β-alanine, 0.5 µmol pantoate and pantothenate synthetase in a total volume of 100 µl. After a suitable incubation period the PS reaction is terminated by the addition of 10 µl of a 0.8 M 2-mercaptoethanol in a 10 % (w/v) solution of sodium dodecylsulfate followed by the addition of 50 µl of a 2.5 % (w/v) solution of ammoniumheptamolybdate in 5 N sulfuric acid. After 20 minutes incubation at room temperature the intensity of the colour complex is determined by measuring the extinction at 620 nm. The amount of pyrophosphate liberated in the PS reaction is determined by reference to a standard curve generated from suitable amounts of pyrophosphate by using the difference of extinction at 620 nm between a complete PS assay mixture and a PS assay mixture lacking pantoate. One unit of PS activity is defined as the amount of enzyme producing 1 nmole of pyrophosphate per minute, and specific activity is expressed as units per milligram of protein.
2. The preferred assay mixture to measure the pyrophosphate liberated in the PS reaction after its conversion with inorganic pyrophosphatase to inorganic phosphate comprises 100 µmol Tris·HCl (pH 8.0), 10 µmol $MgSO_4$, 5 µmol ATP, 10 µmol β-alanine, 0.5 µmol pantoate, 1.0 unit yeast inorganic pyrophosphatase and pantothenate synthetase in a total volume of 100 µl. After a suitable incubation period the PS reaction is terminated either
   a) by the addition of 100 µl of a reagent mixture comprising 62.3 □g malachite green hydrochloride, 1.9 mg ammoniumheptamolybdate and 0.5% (v/v) of a suitable detergent (for example Triton X-100, Tween-80 or Tergitol NPX) in 1.88 N hydrochloric acid. The resulting

colour complex is stabilised by the addition aft r 1 minut of 50 µl of a 26 % (w/v) solution of trisodium citrate dihydrate in water and after an additional 45 minutes incubation at room temperatur the intensity of the colour complex is determined by measuring the extinction at 620 nm. The amount of inorganic phosphate liberated in the PS reaction is determined by reference to a standard curve generated from suitable amounts of inorganic phosphate by using the difference of extinction at 620 nm between a complete PS assay mixture and a PS assay mixture lacking pantoate. Since there are 2 molecules of inorganic phosphate liberated for every molecule of pyrophosphate formed in the PS reaction, one unit of PS activity is defined as the amount of enzyme producing 2 nmoles of inorganic phosphate per minute, and specific activity is expressed as units per milligram of protein; or

b) by the addition of 100 µl of a reagent mixture comprising 3 mg ascorbic acid, 0.5 mg ammoniumheptamolybdate and 1 mg sodium dodecylsulfate in 0.7 N hydrochloric acid. The resulting colour complex is stabilised by the addition after 7 minutes of 50 µl of a 6 % (w/v) solution of trisodium citrate dihydrate in water and after an additional 20 minutes incubation at room temperature the intensity of the colour complex is determined by measuring the extinction at 620 nm. The amount of inorganic phosphate liberated in the PS reaction is determined by reference to a standard curve generated from suitable amounts of inorganic phosphate by using the difference of extinction at 620 nm between a complete PS assay mixture and a PS assay mixture lacking pantoate. Since there are 2 molecules of inorganic phosphate liberated for every molecule of pyrophosphate formed in the PS reaction, one unit of PS activity is defined as the amount of enzyme producing 2 nmoles of inorganic phosphate per minute, and specific activity is expressed as units per milligram of protein.

In order to determine the linear range of the assay used here, recombinant PS purified through the anion exchange chromatography step was assayed by method 2a, using final β-alanine and pantoate concentrations of 10 mM and 0.5 mM, respectively. When various amounts of PS were assayed, a proportional relationship of inorganic phosphate formed and enzyme amount was obtained in between 1 and 4 µg of protein (Figure 7A). Furthermore, when a given amount of PS was assayed for different time periods, a proportional relationship of inorganic phosphate formed and incubation time is obtained in between 0 and 20 minutes of incubation (Figure 7B).

Example 10

Biochemical properties of recombinant *Lotus japonicus* pantothenate synthetase

The recombinant *L. japonicus* enzyme investigated here was found to require pantoate, β-alanine, ATP and Mg^{2+} for activity. The pantoate analogues pantoyl-lactone and ketopantoate were not active as substrates in the place of pantoate. When present at 10-fold excess over pantoate, these analogues did not effect significant inhibition (Table 5).

With 100 mM Tris·HCl buffer optimal PS activity was achieved at pH 8.0. Activity decreases sharply towards more acidic pH's and is nil at pH 7.0, while there is only a slight decrease towards higher pH's with ca. 75% activity left at pH 9.0.

K_m and V_{max} constants for pantoate and β-alanine were determined by measuring the effect of substrate concentration on the reaction rate. Either pantoate or β-alanine were kept at a constant concentration of either 0.5 or 20 mM, and activity assays were carried out using variable concentrations of the other. Plotting the PS activity as a function of substrate concentration according to Lineweaver and Burk (1934) and Eadie (1942) and Hofstee (1959) revealed the kinetic constants as listed in Table 6.

Lotus PS suffers from substrate inhibition by pantoate, which becomes significant at pantoate concentrations of 400 µM or higher. The effect of pantoate concentration on K_m and V_{max} for β-alanine was as expected for pure uncompetitive inhibition, that is both constants decreased with increasing pantoate concentration while their ratio remained constant. K_m over V_{max} for ß-alanine derived from Lineweaver-Burk analysis equalled 0.106 and 0.104 for pantoate concentrations of 20 mM and 0.5 mM, respectively. When the rate equation for uncompetitive substrate inhibition was fitted to the PS activity data for pantoate, values of 42 ± 2 µM and 5.33 ± 0.34 mM were derived for K_S and K'_S, respectively. V_{max} is 11.03 ± 0.19 units in this fit which is equal to a k_{cat} value of 0.625 ± 0.011 sec^{-1}. The K_S and k_{cat} values are very similar to K_m and k_{cat} as derived from linearised plots of activity data.

Using the rate equation for uncompetitive substrate inhibition and the values for K_S and K'_S obtained in the fit, an optimal pantoate concentration of 470 ± 30 µM was calculated.

PS was also assayed in the presence of various compounds that might be expected to possess regulatory properties towards the enzyme. Among these compounds are the intermediates of pantothenate biosynthesis as well as coenzyme A. As coenzyme A plays a prominent role in fatty acid synthesis and degradation, various acyl forms of coenzyme A and free fatty acids were also included. PS was not assayed at optimal substrate concentrations, but pantoate and β-alanine were present at concentrations close to the respective K_m values (0.1 mM and 1 mM). Table 7 lists the compounds tried and their effect on PS activity which is expressed percentage of activity in an assay without additions.

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Table 1

Expression of pantothenate synthetases in *E. coli* AT1371 *(panC)* and wild type strains

E. coli strain	Vector	Specific Activity [U/mg] using Pantoate	OR Pantoyl-lactone	Reference
AT1371 (*panC*)	pSKL *Lotus PS*	8.7	not detected	
AT1371 (*panC*)	pLC *Lotus PS-lacZ*	not detected	not detected	
AT1371 (*panC*)	pRC rice PS-*lacZ*	not detected	not detected	
AT1371 (*panC*)	pYC1 yeast PS	88.0	3.1	this study
AT1371 (*panC*)	pCL *E. coli PS*	957.4	6.0	
AT1371 (*panC*)	pBluescript	not detected	not detected	
K12 (wild type)	---	13.4	---	
K12 (wild type)	---	8.1 - 8.7	---	Cronan *et al.* (1982)
AT1371 (*panC*)	---	< 0.001 (not detected)	---	
B (wild type)	---	4.1	---	Miyatake *et al.* (1979)
W (wild type)	---	1.3	---	Pfleiderer *et al.* (1961)

Table 2

Summary of the purification procedure for recombinant *Lotus japonicus* pantoth nat synth tase.

PS was assayed using pantoate and β-alanine at final concentrations of 1 mM and 10 mM, respectively. Protein was assayed according to the method of Bradford (1976), using the Bio-Rad Protein reagent and microprotein assay in accordance with the manufacturer's instructions. Bovine serum albumine was used to calibrate the assay.

Sample	total protein (mg)	total Units (nmoles/min)	Specific. Activity (U/mg)	recovery (%)	purification
Cleared extract	908.6	27410.2	30.2	(100)	(1)
$(NH_4)_2SO_4$ 30 - 40%	209.7	33030.6	157.5	121	5.2
MonoQ - PS-I	17.8	24160.8	1357.3	88	44.9
MonoQ - PS-II	3.9	4070.7	1038.5	15	34.1
Superose6 - PS-I (a)	2.16	2386.6	1104.9	79 (b)	36.3
Superose6 - PS-II (a)	0.44	400.8	911.0	79 (b)	29.9

(a) Aliquots of the MonoQ - PS-I - pool (2.2 mgs of protein in 0.5 ml) or the MonoQ -PS-II - pool (0.5 mgs of protein in 0.5 ml) were purified further by Superose 6 gelfiltration. PS activity from both PS-I and PS-II eluted in a single peak at identical retention volumes.

(b) The recovery is expressed with respect to the activity loaded onto the gelfiltration column.

Table 3

N-t rminal protein sequences of PS-I and PS-II proteins and predicted sequence for *Lotus japonicus* pantothenate synth tase

Predicted sequence (a):	M A P M V I S D K D E M R K W S R
1° sequence (b):	P M V I S D K D E M R K W S R
2° sequence (b):	A P M V I S D K D E M R K W S R

(a) N-terminal protein sequence predicted from the *Lotus japonicus* ORF for PS (cf. Figure 1.2). The residues underlined correspond to the nucleotide sequence of the PCR primer used in the production of the overexpression clone.

(b) Identical N-terminal protein sequences were obtained for PS-I and PS-II. The molar yield of secondary sequence as compared to primary sequence was ca. 70% in case of PS-I and ca. 30% for PS-II

Tabl 4
Amino acid compositions obtained by amino acid analysis for PS-I and PS-II.

Amino acid	expected value	integer fit of measured mole ratios to expected values	
		PS-I	PS-II
Cys	5	5.64	not determined
Asp	33	35.24	33.67
Thr	6	6.14	6.71
Ser	23	18.79	19.96
Glu	30	24.78	25.19
Gly	26	28.50	26.20
Ala	19	20.02	19.76
Val	33	30.70	32.76
Met	7	7.03	not determined
Ile	20	20.37	19.84
Leu	22	24.56	23.78
Tyr	7	7.01	6.5
Phe	13	15.07	13.01
His	8	7.64	12.11
Lys	20	20.89	22.52
Arg	17	16.97	16.17
Pro	12	11.67	10.82
Trp	5	not determined	not determined
	306 residues		

Tabl 5
Substrate specificity of the recombinant *Lotus japonicus* pantothenat synthetase.

Pantoate	β-alanine	change from standard assay	activity (units)	yield (%)
0.1 mM	1 mM	—	12.51	(100)
0.1 mM	—	—	0	0
—	1 mM	—	0	0
—	1 mM	pantoyl-lactone (1.0 mM)	< 0.1	< 1
—	1 mM	pantoyl-lactone (10 mM)	0.15	1
—	1 mM	ketopantoate (1.0 mM)	0	0
—	1 mM	ketopantoate (10 mM)	0	0
0.1 mM	1 mM	pantoyl-lactone (1.0 mM)	11.53	92
0.1 mM	1 mM	ketopantoate (1.0 mM)	12.18	97

Table 6
Steady state kinetic constants for the recombinant *Lotus* pantothenat synthetase.

Substrate	K_m (a) [μM]	V_{max} (a) [units]	k_{cat} (b) [sec^{-1}]
Pantoate (β-alanine at 20 mM const.)	45 (LB) 44 (EH)	11.36 (LB) 10.98 (EH)	0.63
β-alanine (pantoate at 0.5 mM const.)	990 (LB) 986 (EH)	9.52 (LB) 9.51 (EH)	0.54

(a) Kinetic constants were derived according to Lineweaver-Burk (LB) and Eadie-Hofstee (EH).

(b) Calculation of k_{cat} is based on the V_{max}-mean from LB- and EH-determinations using the known enzyme amount per assay and a molecular weight of 34 kD.

Table 7

Activity of recombinant *Lotus* pantothenate synthetas in th pres nce of various potential eff ctors.

Pantothenate synthetase activity obtained with individual compounds is expressed as a percentage of activity in an assay without effector. The assay was carried out using pantoate and β-alanine at concentrations of 0.1 mM and 1.0 mM, respectively.

Compound	***Concentration [mM]***	***Final activity [%]***
—	—	(100)
a-KIVA	1	89
Ketopantoate	1	97
Pantoyl-lactone	1	92
Pantothenate	1	98
Pyrophosphate	1	78
CoenzymeA	1	109
	0.2	106
	0.1	100
Acetyl-coA	0.100	93
Malonyl-coA	0.100	92
Palmitoleoyl-coA	0.022	114
Oleoyl-coA	0.024	108
Palmitic acid	0.060	115
	0.015	135
	0.002	133
Palmitoleic acid	0.100	100
	0.020	116

Figures

Referring to Figure 1.1 th re is shown a partial restriction map (A), subcloning (B) and nucleotid sequencing (C) of pLC, a *Lotus japonicus* cDNA for pantotheate synthetase. A: The *L. japonicus* pantotheate synthetase cDNA (pLC) was isolated by functional complementation of *E. coli* AT1371 (*pan*C). B represents the sub-clones needed for the DNA sequencing strategy which is summarised in C.

Referring to Figure 1.2 there is shown the nucleotide sequence of the *Lotus japonicus* cDNA for pantothenate synthetase and its predicted amino acid sequence. Figure 1.2 shows the DNA sequence of the 1.33 kb *Eco*RI to *Xho*I insert of pLC (described in Figure 1.1). The open reading frame codes for a polypeptide of 308 amino acids with a predicted molecular mass of 34.2 kDa and with 61% similarity to PS from *E. coli*. The indicated translation start site is putative and the stop codon (TAA) is is translated as "*". This ORF on pLC is in frame with *lacZ* which accounts for expression of functional enzyme in *E. coli* and hence the observed complementation effect

Referring to Figure 2.1 there is shown partial restriction map (A), subcloning (B), and nucleotide sequencing (C) of the rice pantothenate synthetase cDNA. The original cDNA for rice gene, pRC1, was subcloned in order to obtain its complete nucleotide sequence. The arrows indicate the position, direction, and length of individual sequencing runs. The open arrow indicates the EST sequence (GenBank accession no. D25017).

Referring to Figure 2.2 there is shown the nucleotide sequence of the rice cDNA for pantothenate synthetase and its predicted amino acid sequence. The figure shows the DNA sequence of the 1.26 kb *Sal*I to *Not*I insert of pRC1 (Figure 2.1). The ORF encodes a polypeptide of 313 residues with a predicted molecular mass of 33.9 kD. The indicated translation start site is putative, and the stop codon is translated as "*".

Ref rring to Figure 2.3 there is depicted a method for generating the *lacZ*-pantothenate synthetase fusion clone for the expression of rice pantothenate synthetase in *E. coli*. The orientation of the cDNA in pRC1 was changed to yield pRC2 where the open reading frame is under transcriptional control of the *lacZ* promoter. The *lacZ*-PS fusion was generated by deleting four base pairs from pRC2, and the resulting plasmid, pRC, was sequenced to confirm the deletion (data not shown).

Referring to Figure 3.1, the alignment of pantothenate synthetase protein sequences is shown. The PS protein sequences predicted from known (*Lotus japonicus*, *Oryza sativa*, *Escherichia coli*, *Bacillus subtilis*, *Synechocystis sp.)* or putative (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*) genes were aligned using CLUSTAL W(1.5) within the GCG software package. Fully conserved residues are marked "*", functionally conserved ones are marked ".".

lotus: *Lotus japonicus*, rice: *Oryza sativa*, coli: *Escherichia coli* (GenBank P31663), subt: *Bacillus subtilis* (GenBank P52998), syne: *Synechocystis sp.* (GenBank U44896), yeast: *Saccharomyces cerevisiae* (GenBank P40459), pombe: *Schizosaccharomyces pombe* (GenBank Q09673).

Referring to Figure 3.2, the subcloning of yeast pantothenate synthetase for expression in *E. coli* is depicted. **A:** The λ bacteriophage clone IPM4950 was obtained from the Sanger Centre, Hinxton Hall, Cambridge, UK, where the yeast PS sequence had been generated. **B+C:** Yeast *panC* was subcloned in two steps to yield plasmid clone pYC1 where the gene is placed under transcriptional control of the *lac* promoter. The position of the ORF is indicated by arrows. A T3-primed sequencing reaction using pYC1 as template confirmed the identity of the *Eco*RV-*Hind*III insert of the plasmid.

Referring to Figure 3.3, there is shown the nucleotide sequence of the *Saccharomyces cerevisiae* genomic DNA fragment for pantothenate synthetase and its predicted amino acid sequence. Figure 3.3 shows the nucleotide sequence of the 1.5 kb *Eco*RV to *Hind*III genomic DNA fragment of *S.*

cerevisiae that forms the insert of pYC1. The predicted amino acid sequence of yeast PS appears below the open reading frame. A Shine-Dalgarno-like sequence upstream of the translation initiation codon that may fortuitously serve as a RBS in *E. coli* is underlined. § - *Eco*RV; ¶ - *Hind*III.

Referring to Figure 4 there is shown the inverse PCR product of *Lotus japonicus* genomic regions flanking *panC*. **A:** Schematic representation of the iPCR product cloned into pCRII. The EcoRI.EcoRI insert was sequenced using T7 and M13 reverse primers. Both sequence runs were performed in duplicate and spanned ca. 600 bases each. **B:** Nucleotide sequence of the cloned iPCR product. The indicated matches with *panC* cDNA mean identical sequences. Positions corresponding to the first base (5' - ¶) or the last base (3' - §) of the *panC* cDNA are marked. Within the 5' flanking genomic sequence, there is a stop-codon in frame with the *panC* ORF.

Referring to Figure 5, the expression cassette PCR of *Lotus japonicus* pantothenate synthetase is shown.

Referring to Figure 6, there is shown the anion exchange chromatographs of recombinant *Lotus japonicus* pantothenate synthetase is shown. A sample of ammonium sulphate precipitated and dialysed PS was subjected to anion exchange chromatography on a MonoQ HR10/10 column as described in Example 7. **A:** PS activity profile. Fractions 29 through 32 were pooled to give sample PS-I, and fractions 57 through 60 were pooled to give sample PS-II. **B:** Protein elution profile followed by continous measurement of A_{280} and potassium chloride gradient employed.

Referring to Figure 7 the results of a high-throughput assay for recombinant *Lotus japonicus* pantothenate synthetase is depicted graphically. Graph A shows the effect of enzyme concentration on the reaction rate. Specific amounts of MonoQ purified PS-I were assayed as described in Example 9, method 2a. The activity-response is proportional in a range from 1 to 4 µg PS

per assay. Graph B shows the time course of inorganic phosphate formation. 1.2 mg ( ), 2.4 mg (■), and 3.0 mg (▲) of MonoQ purified PS-I wer assayed as described in Example 9, method 2a. The activity-response is proportional in a range from 0 to 20 minutes of incubation.

Claims

1. An isolated DNA molecule encoding a protein from a plant, which protein has pantothenate synthetase activity.

2. An isolated DNA molecule according to claim 1, wherein the plant is *Lotus japonicus.*

3. An isolated DNA molecule according to claim 2, wherein the DNA molecule encodes a protein comprising the amino acid sequence set forth in Figure 1.2.

4. An isolated DNA molecule according to claim 3, wherein the DNA molecule comprises the nucleotide sequence set forth in Figure 1.2.

5. An isolated DNA molecule according to claim 1, wherein the plant is *Oryza sativa.*

6. An isolated DNA molecule according to claim 5, wherein the DNA molecule encodes a protein comprising the amino acid sequence set forth in Figure 2.2.

7. An isolated DNA molecule according to claim 6, wherein the DNA molecule comprises the nucleotide sequence set forth in Figure 2.2.

8. A non-naturally occuring chimeric gene comprising a promoter operably linked to a DNA molecule encoding a protein from a plant having pantothenate synthetase activity.

9. A non-naturally occuring chimeric gene according to claim 8 wherein the protein comprises an amino acid sequence selected from the group set forth in Figure 1.2 and Figure 2.2.

10. A recombinant vector comprising the chimeric gene of any of claims 8 to 9 wh rein the v ctor is capable of being stably transformed into a host cell.

11. A host cell stably transformed with the vector of claim 10 wherein the host cell is capable of expressing the DNA molecule.

12. A host cell according to claim 11 selected from the group consisting of a bacterial cell, a yeast cell and an insect cell.

13. A method of producing a protein having pantothenate synthetase activity in a host organism comprising,

a) inserting a DNA sequence encoding a protein having antothenate synthetase activity into an expression casette designed for the chosen host;

b) inserting the resultant molecule, containing the individual lements linked in proper reading frame, into a vector capable of being transformed into the host cell;

c) growing the thus transformed host cell in a suitable culture medium; and

d) isolating the protein product either from the transformed cell or the culture medium or both and purifying it.

14. A method for assaying a protein having pantothenate synthetase activity comprising,

a) incubating the protein in a suitable reaction mixture in which the protein is capable of catalyzing the conversion of pantoate, ß-alanine and ATP to pantothenate, AMP and pyrophosphate; and

b) determining the amount of pyrophosphate generated by the catalytic activity of pantothenate synthetase by a colorimetric technique.

15. A method for assaying a prot in having pantoth nate synth tas activity comprising, incubating the protein in a suitable reaction mixture in which the prot in is capabl of catalyzing the conversion of pantoate,

a) ß-alanine and ATP to pantothenate, AMP and pyrophosphate; and

b) converting the pyrophosphate formed by the catalytic activity of pantothenate synthetase into inorganic phosphate by the catalytic activity of an inorganic pyrophosphatase; and

c) determining the amount of inorganic phosphate generated by the catalytic activity of the inorganic pyrophosphatase by a colorimetric technique.

16. A method according to claim 15 wherein the inorganic pyrophosphatase is yeast pyrophosphatase.

17. A method for assaying a chemical for the ability to inhibit the activity of a pantothenate synthetase enzyme from a plant comprising,

a) incubating the pantothenate synthetase enzyme in a first reaction mixture under conditions specified in claim 14 or claim 15;

b) combining the chemical and the pantothenate synthetase enzyme in a second reaction mixture under the same conditions as in the first reaction mixture; and

c) measuring the amount of either pyrophosphate or inorganic phosphate in the first and second reaction mixtures;

wherein the chemical is capable of inhibiting the activity of the pantothenate synthetase enzyme if the amount of either pyrophosphate formed or inorganic phosphate formed in the second reaction mixture is significantly less than the amount of either pyrophosphate formed or inorganic phosphate formed in the first reaction mixture.

18. The use as herbicides of compounds which inhibit pantothenate synthetase.

19. Herbicidal composition, comprising one or more active ingredients which show significant panthotenate synthetase inhibition in an assay according to claim 17.

20. A nucleotide probe capable of specifically hybridizing to a plant pantothenate synthetase gene or mRNA, wherein the probe comprises a contiguous portion of the coding sequence for a pantothenate synthetase from a plant at least 10 nucleotides in length.

21. A method of producing a DNA molecule comprising a DNA portion encoding a protein having pantothenate synthetase activity comprising,

a) preparing a nucleotide probe capable of specifically hybridizing to a plant pantothenate synthetase gene or mRNA, wherein the probe comprises a contiguous portion of the coding sequence for a pantothenate synthetase from a plant at least 10 nucleotides in length;

b) probing for other pantothenate synthetase coding sequences in populations of cloned genomic DNA fragments or cDNA fragments from a chosen organism using the nucleotide probe prepared according to step a); and

c) isolating a DNA molecule comprising a DNA portion encoding a protein having pantothenate synthetase activity.

22. The use of a nucleotide probe according to claim 19 to amplify pantothenate synthetase coding sequences from a chosen organism via the process of polymeras chain reaction.

Figure 1.1

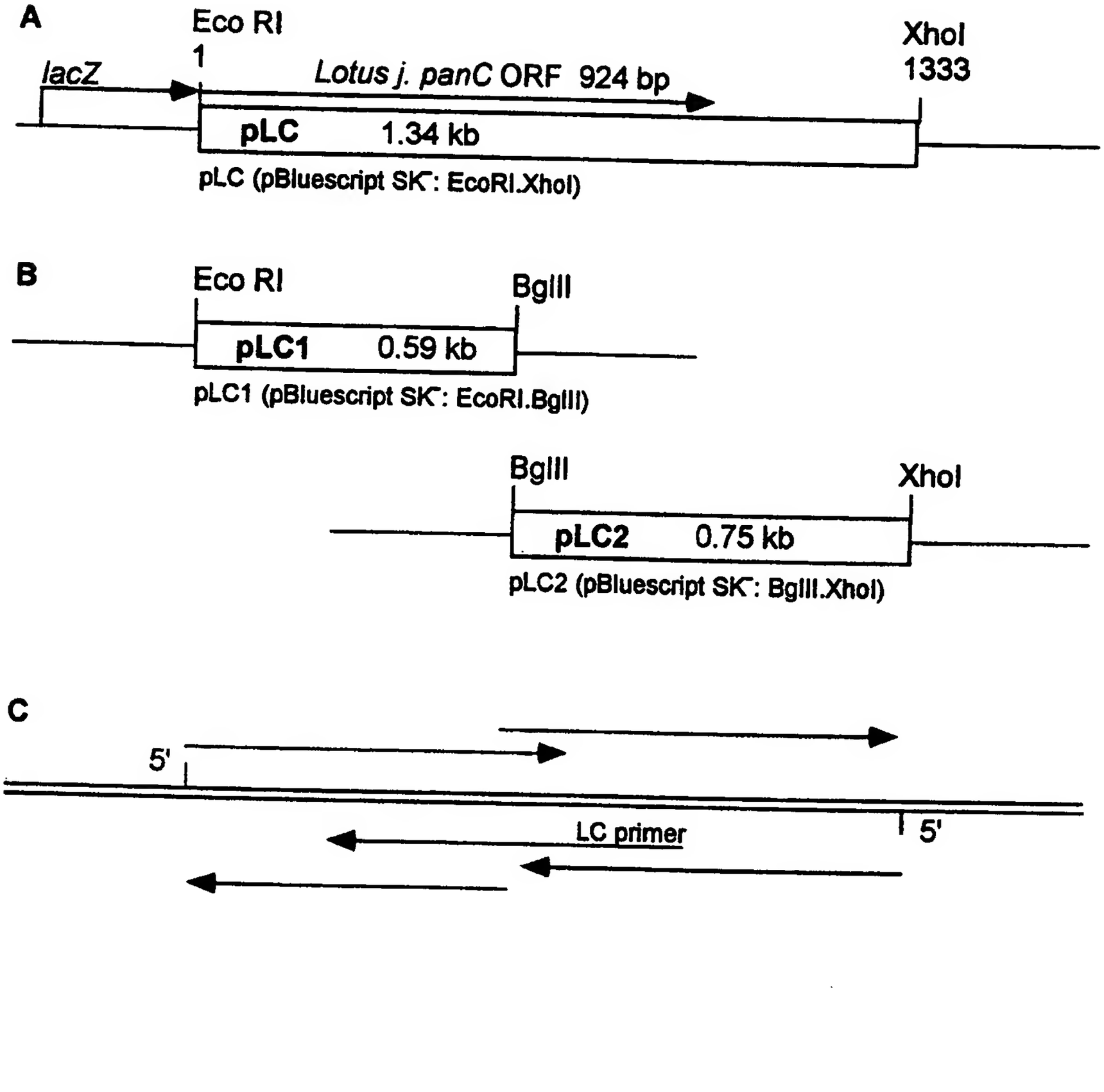

A
Eco RI
1
lacZ
Lotus j. panC ORF 924 bp
XhoI
1333
pLC 1.34 kb
pLC (pBluescript SK⁻: EcoRI.XhoI)
B
Eco RI
BglII
pLC1 0.59 kb
pLC1 (pBluescript SK⁻: EcoRI.BglII)
BglII
XhoI
pLC2 0.75 kb
pLC2 (pBluescript SK⁻: BglII.XhoI)
C
5'
LC primer
5'

Figure 1.2

EcoRI *start*

GAATTCGGCACGAGCTCCAATGGCACCAATGGTGATATCTGATAAGGACGAGATGCGGAAATGGTCAAGGTCCATGCGATCCCAAGGCAAGCTCATCGCC -
M A P M V I S D K D E M R K W S R S M R S Q G K L I A

CTCGTTCCCACCATGGGCTTCCTTCACGAAGGCCACCTTTCTCTCGTCAGAGACGCTCACAACCACGCTGACCTCGTCGCCGTCTCAATCTATGTCAACC -
L V P T M G F L H E G H L S L V R D A H N H A D L V A V S I Y V N P

CTGGCCAGTTTTCCCCGACCGAGGACCTTTCCGCATACCCTTCTGATTTTCAAGGTGATCTCCAAAAACTCATGTCTGTTCCTGGTGGTGTTGATGTTGT -
G Q F S P T E D L S A Y P S D F Q G D L Q K L M S V P G G V D V V

TTTCCACCCCCACAATTTGTATGATTACGGTGGTGATGGCGGTGATGCTGTGGCGGAGTGTGGTGGTGATGGGGTGGTGTCTTGTGTTGATAGGAGGAGT -
F H P H N L Y D Y G G D G G D A V A E C G G D G V V S C V D R R S

GGTTTTGGGCATGAAACTTGGGTTAGAGCTGAGAAGCTGGAGAAACCCCTTTGTGGGAAGAGTAGGCCTGTTTTCTTTAGAGGGGTTGCCACCATTGTTA -
G F G H E T W V R A E K L E K P L C G K S R P V F F R G V A T I V T

BglII

CCAAGTTGTTTAATATTGTGGAGCCTGATGTTGCTGTGTTTGGGAAGAAGGACTATCAGCAATGGAAAATTATTCAGAGAATGGTTCGAGATCTTGATTT -
K L F N I V E P D V A V F G K K D Y Q Q W K I I Q R M V R D L D F

TTCCATAAAAGTGATAGGTTCTGAAGTAATACGTGAGAAAGATGGCCTAGCAATGAGTTCCCGTAATGTGTACCTATCACCTGAAGAGAGGGAAAAGGCA -
S I K V I G S E V I R E K D G L A M S S R N V Y L S P E E R E K A

GTATCTATAAATAAATCATTGTTTAGAGCTAAATCGGCAGCAGAAGATGGACAGATACATTGTGAGAAATTGATAAACTTGGTCGTGCAAAGTATCACCG -
V S I N K S L F R A K S A A E D G Q I H C E K L I N L V V Q S I T E

AAGCTGGTGGAAGGATTGATTATGCTGAGATTGTTGATCAAAATAATTTGGAGAAAGTGGAATGGATCAAGGGTCCTGTTGTCTTCTGTGTTTCTGCATG -
A G G R I D Y A E I V D Q N N L E K V E W I K G P V V F C V S A W

GTTTGGGAAAGCCAGGCTTATAGACAACATAGAAATCAACTTGTAAATGGAAGTAAGATTGATCTAACCTTGTGAATAATCTCAGACATGGACCATATGA -
F G K A R L I D N I E I N L *

TTAGTAGTTCTGGCATTTCATGGGGTATAGACTTCATTCTACAAGCCATGATATGACTACTTGTAGATGTATTTTACTACCTCATGAAATTCTAGGAGCT -

GCTTCTATTTGTTGGTGATGGTATAATATTTTGCAGAGCCACCACTCCAGAGGAAAACAAAATTAGAGAAATCTTGCTTATGTATCAAAGTGCCCCAGGT -

TTACTCATTAATCTAGATAAATCTGAGCTTTCTTTAGGCTGATGTACGCCTAGAGATAGACAAACATAATTCTGGTGCTGGATAAAATTAACGCATTGGA -

XhoI

TTCCCATTTGAAATAAAAAAAAAAAAAAAAAACTCGAG

Figure 2.1

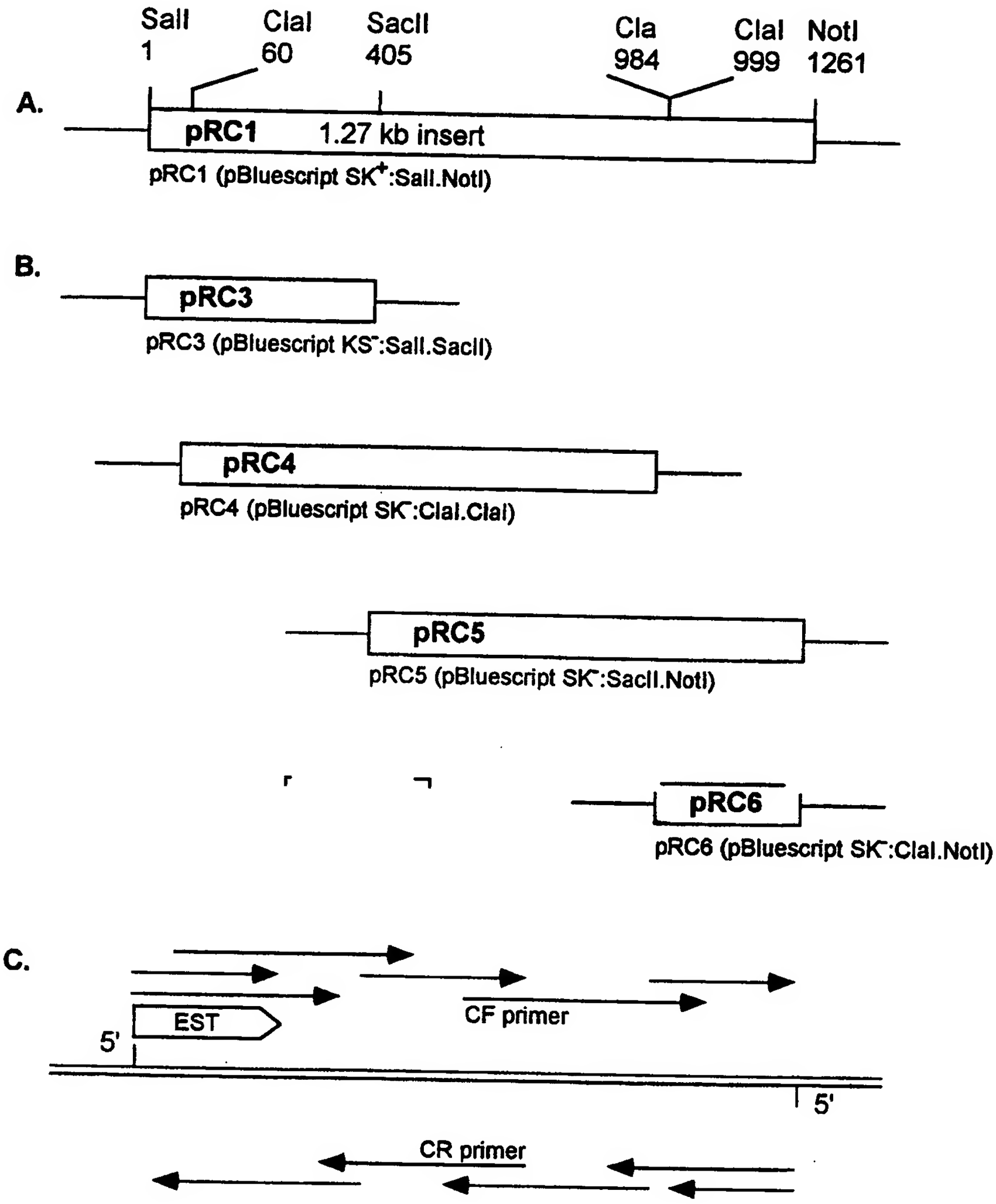

A.
SalI
1
ClaI
60
SacII
405
Cla
984
ClaI
999
NotI
1261
pRC1
1.27 kb insert
pRC1 (pBluescript SK+:SalI.NotI)
B.
pRC3
pRC3 (pBluescript KS-:SalI.SacII)
pRC4
pRC4 (pBluescript SK-:ClaI.ClaI)
pRC5
pRC5 (pBluescript SK-:SacII.NotI)
pRC6
pRC6 (pBluescript SK-:ClaI.NotI)
C.
EST
5'
CF primer
5'
CR primer

Figure 2.2

SalI *ClaI* *start*

GTCGACCCACGCGTCCGGTTTCTCCCTGTCCACTTCTGTCCGATTCCTCCTCACCTCTTATCGATTGGACGACCATGGCGGCGCCGCGCGAGCCGGAGGT - 100
P V S P C P L L S D S S S P L I D W T T M A A P R E P E V

GATCCGCGACAAGGCGGCGATGCGCGCATGGTCGCGCCGCCGTCGCGCCGAGGGCAAGACCGTCGCGGTCGTACCCACCATGGGCTACCTCCACCAAGGC - 200
I R D K A A M R A W S R R R R A E G K T V A V V P T M G Y L H Q G

CACCTCTCCCTCATCTCCGCCGCCGCCGCCGCCGCCTCCGCTGATCCCGTCGCCATCGTCGTCACCATCTACGTCAACCCCAGCCAGTTCGCGCCCTCAG - 300
H L S L I S A A A A A A A S A D P V A I V V T I Y V N P S Q F A P S E

AGGACCTCGCCACCTACCCTTCCGACTTCGCCGGTGACCTCCGCAAGCTCGCCTCCACCGGCGTCGTGGATGCCGTCTTCAACCCCCCTGACCTCTACGT - 400
D L A T Y P S D F A G D L R K L A S T G V V D A V F N P P D L Y V

SacII

CCGTGGCGCCGGTCGCCGCGGGGCCGGCTCCGGAGGCGCGATCTCCTGCCTGGAGGAGGCGGCCGGGGATGGGCACGAGACGTGGGTTCGGGTGGAGCGA - 500
R G A G R R G A G S G G A I S C L E E A A G D G H E T W V R V E R

TTGGAGAAGGGATTGTGCGGGGCCAGCCGTCCCGTGTTCTTCCGAGGCGTGGCCACCATAGTCTCCAAGCTGTTTAACATCATCGAGCCGGATGTTCCTG - 600
L E K G L C G A S R P V F F R G V A T I V S K L F N I I E P D V P V

TGTTCGGGAAGAAGGATTATCAGCAGTGGCGCGTCATCTTGCCGTATTGGTCGGGACTTGATTTTGGCATAGAGATAATGGGATCAAGAAATTGTGCGAG - 700
F G K K D Y Q Q W R V I L P Y W S G L D F G I E I M G S R N C A R

AACTGATGGTCTTGCCATGAACTCCCGGAATGTGCACCTATCACGCGAGGAAGGGAAAAAGGCATTATCCATCAGTAGATCACTGGTTGATGCTAGAACT - 800
T D G L A M N S R N V H L S R E E G K K A L S I S R S L V D A R T

GGCGCCTTGAAGGGAAACACTGATTCCAAACAAATCAAAAACAAAATAGTACAGACACTAACTGAAACTGGCGGTCAGGTTGACTATGTTGAGATCGTGG - 900
G A L K G N T D S K Q I K N K I V Q T L T E T G G Q V D Y V E I V E

ClaI

AGCAAGAAAGTTTGGTCCCTGTAGAACAGATCGACGGCCCTGTGGTCATTTGCGTTGCGGCGTGGTTTGGAAAGGTCAGGCTGATCGATAATATCGAAAT - 1000

Q E S L V P V E Q I D G P V V I C V A A W F G K V R L I D N I E I

ClaI

CGATACACGATCCTGAGGTTTTGGGGGGATTCACTTGCTGTCTGCTGTGACCTTGGCATTGCGTTTGAAATACCTTTTGTTTCGCGTGATGATTCGCGTC - 1100

D T R S *

ATGTTGTACGCTGTAACAATCACAGAGAGAAAATATGCAGGAGTACACTGACTGAAGGCAAATTTATAAGTACAAACTGTAGAGGCCTGATGCTGTAACA - 1200

NotI

GGGGAAATCATGCTTGTTGATTACAGATTCCGCTGAAAAAAAAAAAAAAAAAAAAGGGCGGCCGC - 1264

Figure 2.3

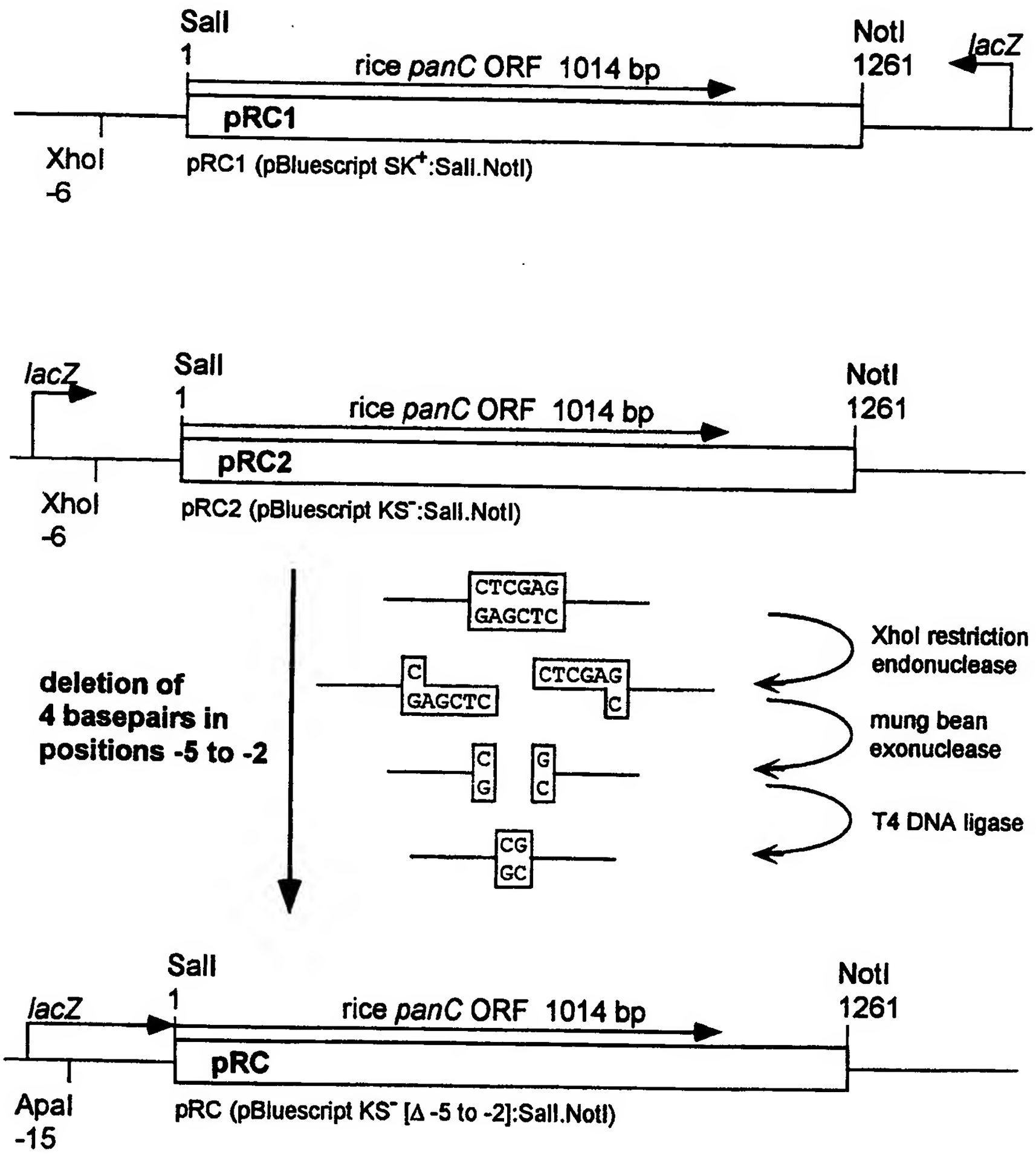

SalI
1
rice panC ORF 1014 bp
NotI
1261
lacZ
pRC1
XhoI
-6
pRC1 (pBluescript SK+:SalI.NotI)
lacZ
SalI
1
rice panC ORF 1014 bp
NotI
1261
pRC2
XhoI
-6
pRC2 (pBluescript KS-:SalI.NotI)
deletion of
4 basepairs in
positions -5 to -2
CTCGAG
GAGCTC
C
GAGCTC
CTCGAG
C
C
G
G
C
CG
GC
XhoI restriction
endonuclease
mung bean
exonuclease
T4 DNA ligase
SalI
1
lacZ
rice panC ORF 1014 bp
NotI
1261
pRC
ApaI
-15
pRC (pBluescript KS- [Δ -5 to -2]:SalI.NotI)

Figure 3.1

```
lotus     ------------------------------------PMAPMVISDKDEMRKWS---RSMRSQGKLIALVP
ric       ------------PVSPCPLLSDSSSPLIDWTTMAAPREPEVIRDKAAMRAWS---RRRRAEGKTVAVVP
coli      ------------------------------------VLIIETLPLLRQQI---RRLRMEGKRVALVP
pombe     ------------------------------------MQVLKEKLLIHQQV---DNWRKDGNRIAFVP
subt      ------------------------------------MRQITDISQLKEAI---KQYHSEGKSIGFVP
syne      ------------------------------------VQVFRTIAGLQTY----LRQAGRGKTVGLVP
yeast     ---MNISRCSVHIYVYKKWCVLILHCFIQVFILHIHIELMKIFHTVEEVVQWRTQELRETRFRETIGFVP
                                                          .          .  **

lotus     TMGFLHEGHLSLVRDAHNHAD----LVAVSIYVNPGQFSPTEDLSAYPSDFQGDLQKLMSVPGGVDVVFH
rice      TMGYLHQGHLSLISAAAAAASADPVAIVVTIYVNPSQFAPSEDLATYPSDFAGDLRKLAS-TGVVDAVFN
coli      TMGNLHDGHMKLVDEAKARAD----VVVVSIFVNPMQFDRPEDLARYPRTLQEDCEKLNKR--KVDLVFA
pombe     TMGNLHEGHFSLVREAKRHAE----KVVVSIFVNPMQFNNPQDLLLYPRTMDQDCSQLQNL--GVDLVYA
subt      TMGFLHEGHLTLADKARQEND----AVIMSIFVNPAQFGPNEDFEAYPRDIERDAALAENA--GVDILFT
syn       TMGSLHAGHGSLLKRAVAEMD----LVVLSIFVNPLQFGPGEDLEKYPRDFDGDRQWAESL--GVAVIFA
yeast     TMGCLHSGHASLISQSVKENT----YTVVSIFVNPSQFAPTEDLDNYPRTLPDDIKLLESL--KVDVLFA
          *** ** **  *   .             ..*.*** **   .*   **     *          *  ..

lotus     P----HNLYDYG------GDGGDAVAECGGDGVVSCVDRRSGFGHETWVRAEKLEKPLCGKSRPVFFRGV
rice      P-----PDLYVRG------AGRRGA----GSGGAISCLEEAAGDGHETWVRVERLEKGLCGASRPVFFRGV
coli      PSV--KEIYPNG------TET-----------------------HTYVDVPGLSTMLEGASRPGHFRGV
pombe     PTV--EELYPEG------SQD-----------------------ITFVDVPKLSTMLEGASRPGHFRGV
subt      PDA--HDMYP--------GEK-----------------------NVTIHVERRTDVLCGRSREGHFDGV
syne      PTVTDLGIDAKG------DQT-----------------------TVLPPPAMTEVLCGAHRPGHFQGV
yeast     PNA--HVMYPQGIPLDIEEQK-----------------------GPFVSVLGLSEKLEGKTRPNFFRGV
          *      .                                     .          * *  *   * **

lotus     ATIVTKLFNIVEPDVAVFGKKDYQQWKIIQRMVRDLDFSIKVIGSEVIREKDGLAMSSRNVYLSPEEREK
rice      ATIVSKLFNIIEPDVPVFGKKDYQQWRVILPYWSGLDFGIEIMGSRNCARTDGLAMNSRNVHLSREEGKK
```

```
coli        STIVSKLFNLVQPDIACFGEKDFQQLALIRKMVADMGFDIEIVGVPIMRAKDGLALSSRNGYLTAEQRKI
pombe       TTVVSKLFHIVNPDVACFGEKDFQQVAIIKKMVRDLNFFIEIIQVPIVRADDGLALSSRNGYLTSEERKI
subt        AIVLTKLFNLVKPTRAYFGLKDAQQVAVVDGLISDFFMDIELVPVDTVREEDGLAKSSRNVYLTAEERKE
syne        ATIVTKLFTIVCPDVAYFGAKDAQQLAIIRRLVQDLNLTVTIRSCATVREESGLAMSSRNQYLSPIEKEQ
yeast       ATVVTKLFNIVMADVAYFGQKDIQQFIVLQCMVDELFVNTRLQMMPIVRNNNGLALSSRNKYLCPESLKI
                . ...*** ..      ** ** **  ..            .          ***  ***  *.

lotus       AVSINKSLFRAKSAA----EDGQIHCEKLINLVVQS----ITEAGGRIDYAEIVDQNNLEKVEWIKG---
rice        ALSISRSLVDARTGA----LKGNTDSKQIKNKIVQT----LTETGGQVDYVEIVEQESLVPVEQIDG---
coli        APGLYKVLSSIADKL----QAGERDLDEIITIAGQE----LNEKGFRADDIQIRDADTLLEVSETSK---
pombe       APNLYKILKKLAQEL----SNGNGDLEKLIAETNTE----LSRCRFIPDQLEICDSTTLEPFTAGTK---
subt        APKLYRALQTSAELV----QAGERDPEAVIKAAKDI----IETTSGTIDYVELYSYPELEPVNEIAG---
syne        ATVLYRSLQAAPTAI-----SSRRSPS----------FCFVDRHPGRFGRGTVLSRCNICNWWKLTPC--
yeast       SENLYRGLKAAENAIRRLAPGGRLSRSEIIDTVTQIWAPYVDSHDFKIDYVSLADFKTLDELSDVENTSE
              .  . . *                        .          .     .

lotus       PVVFCVSAWFGK---------ARLIDNIEINL------
rice        PVVICVAAWFGK---------VRLIDNIEIDTRS----
coli        RAVILVAAWLGD---------ARLIDNKMVELA-----
pombe       NVVILAAAWLGK---------ARLIDNIQTTIN-----
subt        KMILAVAVAFSK---------ARLIDNIIIDIREMERI
syne        -QPITWNITGPKSCFNGDR---RLCG------------
yeast       QQPIVISCAVYVTDREKPDTVVRLIDNIVI--------
                                  **
```

Figure 3.2

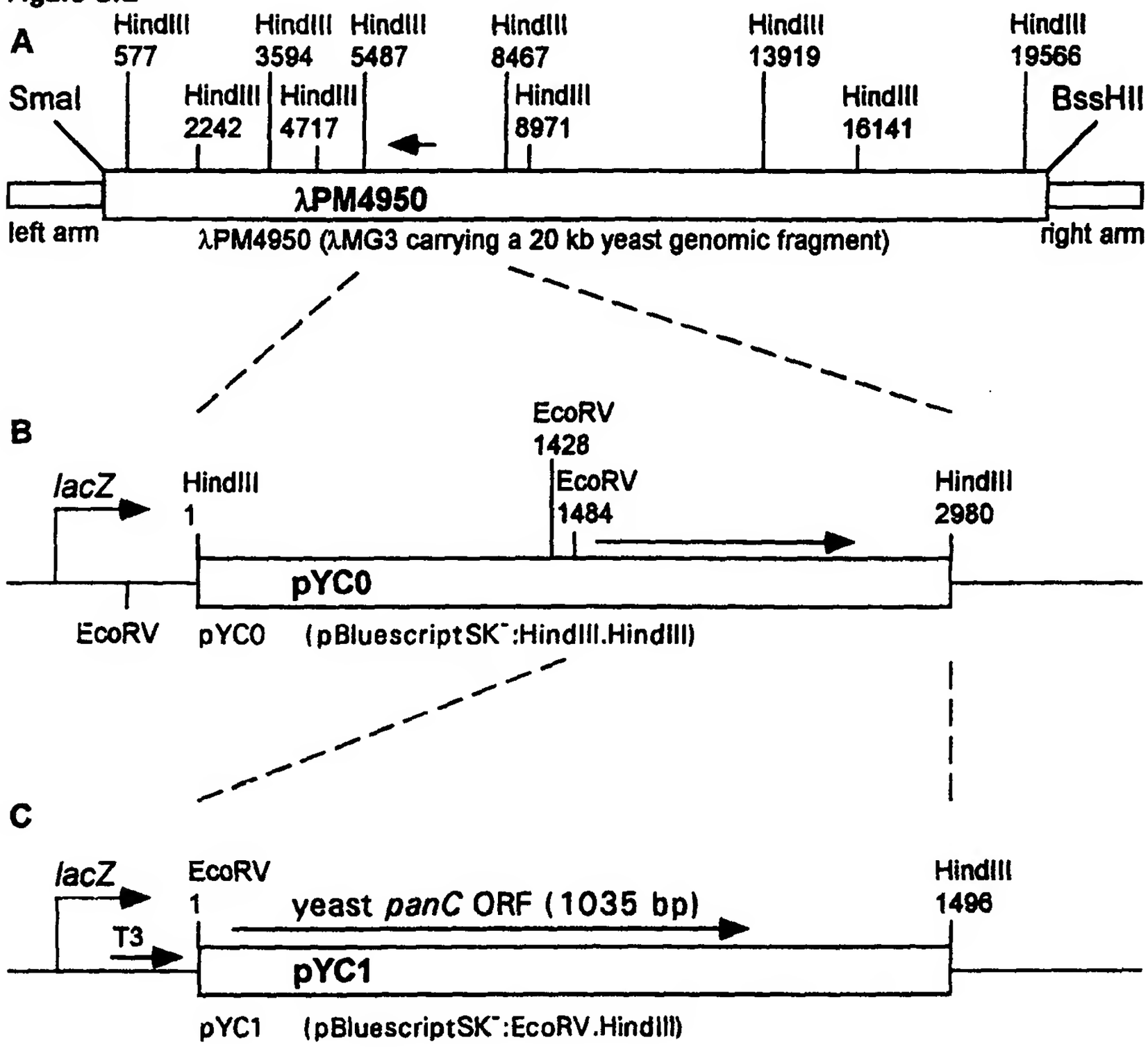

A
HindIII 577
HindIII 3594
HindIII 5487
HindIII 8467
HindIII 13919
HindIII 19566
SmaI
HindIII 2242
HindIII 4717
HindIII 8971
HindIII 16141
BssHII
λPM4950
left arm
λPM4950 (λMG3 carrying a 20 kb yeast genomic fragment)
right arm
B
EcoRV 1428
lacZ
HindIII 1
EcoRV 1484
HindIII 2980
pYC0
EcoRV pYC0 (pBluescriptSK⁻:HindIII.HindIII)
C
lacZ
EcoRV 1
yeast panC ORF (1035 bp)
HindIII 1496
T3
pYC1
pYC1 (pBluescriptSK⁻:EcoRV.HindIII)

Figure 3.3 s

```
GATATCCGGGTAAATGTTACTTTAACGAGTTTTTTTCTTTTGTCATATTTTCCAACAAGAGATTAATGAACATTTCTCGATGTAGCGTACATATTTATGT
                                                                  M  N  I  S  R  C  S  V  H  I  Y  V

GTACAAGAAGTGGTGTGTGTTGATACTGCACTGTTTTATACAAGTTTTTATACTGCATATCCATATAGAATTGATGAAAATCTTCCATACTGTCGAAGAA
 Y  K  K  W  C  V  L  I  L  H  C  F  I  Q  V  F  I  L  H  I  H  I  E  L  M  K  I  F  H  T  V  E  E

GTTGTTCAATGGAGAACACAGGAGCTGAGGGAAACTAGATTTAGAGAAACTATTGGGTTCGTTCCCACAATGGGTTGCCTGCATTCGGGTCACGCTAGTT
V  V  Q  W  R  T  Q  E  L  R  E  T  R  F  R  E  T  I  G  F  V  P  T  M  G  C  L  H  S  G  H  A  S  L

TGATCTCGCAGTCTGTGAAGGAAAACACCTATACTGTGGTCAGTATATTTGTAAATCCCTCCCAGTTTGCGCCAACGGAAGATCTAGATAACTATCCTCG
  I  S  Q  S  V  K  E  N  T  Y  T  V  V  S  I  F  V  N  P  S  Q  F  A  P  T  E  D  L  D  N  Y  P  R

AACTTTGCCAGACGACATCAAATTGCTTGAGTCGTTGAAGGTGGATGTTCTATTTGCTCCTAATGCACACGTGATGTATCCACAGGGAATTCCGCTCGAC
 T  L  P  D  D  I  K  L  L  E  S  L  K  V  D  V  L  F  A  P  N  A  H  V  M  Y  P  Q  G  I  P  L  D

ATAGAAGAGCAGAAAGGCCCTTTTGTTAGTGTTCTTGGATTGAGTGAAAAATTAGAGGGGAAGACGAGACCTAACTTCTTTAGGGGCGTGGCAACTGTCG
I  E  E  Q  K  G  P  F  V  S  V  L  G  L  S  E  K  L  E  G  K  T  R  P  N  F  F  R  G  V  A  T  V  V

TGACTAAACTATTCAATATCGTTATGGCGGATGTGGCTTATTTTGGGCAGAAGGACATTCAACAGTTCATTGTTTTACAGTGTATGGTGGACGAACTGTT
  T  K  L  F  N  I  V  M  A  D  V  A  Y  F  G  Q  K  D  I  Q  Q  F  I  V  L  Q  C  M  V  D  E  L  F

TGTTAATACAAGGCTACAAATGATGCCTATTGTAAGAAACAATAATGGACTGGCTCTGAGTAGTAGAAACAAATATCTTTGTCCAGAGTCTTTAAAGATC
 V  N  T  R  L  Q  M  M  P  I  V  R  N  N  N  G  L  A  L  S  S  R  N  K  Y  L  C  P  E  S  L  K  I

TCTGAAAACCTTTACCGCGGGCTGAAAGCTGCGGAAAATGCTATTAGGAGACTAGCACCAGGGGGACGTCTCTCCAGATCAGAAATCATCGATACTGTGA
S  E  N  L  Y  R  G  L  K  A  A  E  N  A  I  R  R  L  A  P  G  G  R  L  S  R  S  E  I  I  D  T  V  T

CTCAAATATGGGCACCCTACGTTGATTCCCACGATTTCAAAATCGACTATGTTTCCTTAGCAGATTTTAAGACTCTTGATGAACTCTCCGATGTTGAAAA
  Q  I  W  A  P  Y  V  D  S  H  D  F  K  I  D  Y  V  S  L  A  D  F  K  T  L  D  E  L  S  D  V  E  N

CACCAGCGAACAGCAGCCAATAGTCATTAGTTGTGCTGTATACGTGACTGACCGCGAAAAACCCGATACGGTCGTCAGACTAATAGATAACATCGTTATT
 T  S  E  Q  Q  P  I  V  I  S  C  A  V  Y  V  T  D  R  E  K  P  D  T  V  V  R  L  I  D  N  I  V  I

TAAACTAGGTGATTGGGCCTTCCCGTGTCTGTGTTGCAGTATATACCACTCTTATACAGTATGCACGATATTCTTTTAAACCAACAACGGGATGATAGAT
*
```

TTCACGCTTGATGACTTTTTTTTTAGACGGCTGAAGGGACGACATCCCCATCGCTCAAAACACAAATATGGAAAGGACAAATCGTCTTTCACAGTTTGCA

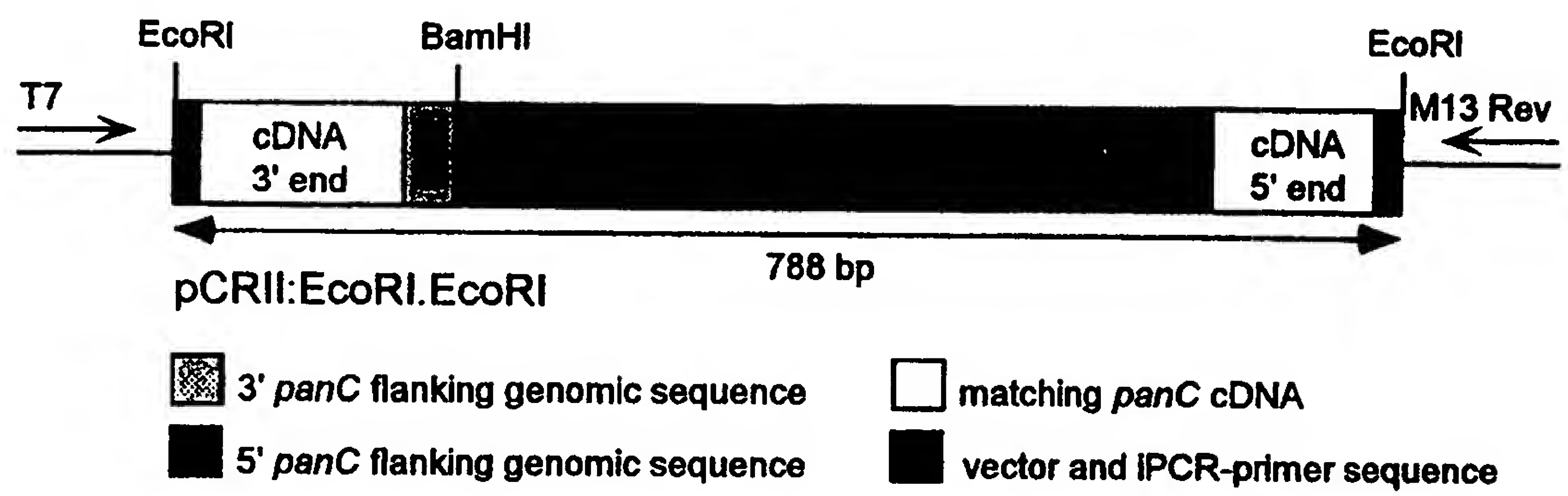
TAGTAAAAGCAAAGTTTATACTACTTCAGCAAAGTTGAAGTTGTTTGGCACTTGTTTCGTGCTTTCTCAAATATCTTAGATCACCGTCTGTCTAGAGCAT

1

ATATCTATTGTTTGACGCACCCCTTTTACAAAAAAAAAAAAAAGAAACAGATCTATTAAGTAATAAAAAAGTTATTTAGGAAATAAGGTGCAGTAAGCTT

Figure 4

A



B

```
                EcoRI                / matching panC cDNA (bases 1080- 1314)  ---> ...
  1     5'  GAATTCGGCTTCATCAAGCTTATGTATCAAAGTGCCCCAGGTTTACTCATTAATCTAGATAAATCTGAGCTTTCTTTAGG
                         Li3 primer

                                                       3' end of cDNA (without polyA) $ ...
 81         CTGATGTACGCCTAGAGATAGACAAACATAATTCTGGTGCTGGATAAAATTAACGCATTGGATTCCCATTTGAAATATCT

              3' flanking  ---> BamHI <---  5' flanking genomic sequence ...
161         TGGTCTCCCTATTTTATAGGAGGATCCAACGGCAGAGAACCTAGGTTCTCATGACCCCTCTTGAGGGCCATCCTAGCTTC

            ...
241         GGGATCTCATATAAAAGGTCCCTTACAACAAGGGGCCAATGTAACTTCTCTTAGCTCAATTACTTTGATAACTTAGACTT

            ...
321         TTCATCCTGCTGAACCAGAATTGACTTAGGCATCAAAGTGGTTTGCACGACCCCCTCCCGGGCTTACCTGTACGTTGCAG
```

```
          ...
401       ACTACCTCTCTCCAGTCGTTCGCTCGAGCTCCAACCTCCCTCACCCCCGTTCCCCTTCCCTCCTACCCCTTCGTGGGTAG

          ...
481       TCTTGGTCGAATCTTCCCGGATCAATATCATATATGATATCATCATCATTTCAATAGAATGAAGCGCCACCTATCTATTT

          ...
561       GCTTCATCAAAAGCCTTCTTTTGCAAGAGTTCCCATTTGTTCTTATCACCTTCACGTTCAACTAGCTTTACACTTTTTCG

          ...                          ... ¶ 5' start of cDNA ---> ...
641       ACATTCCCAATAACAACACCAGAACCCTCCTCCAATGGCACCAATGGTGATATCTGATAAGGACGAGATGCGGAAATGGT
                    stop                          start panC ORF
                                                  M  A  P  M  V  I  S  D  K  D  E  M  R  K  W  S

                    ... matching panC cDNA (bases 1 - 101) \                    EcoRI
721       CAAGGTCCATGCGATCCCAAGGCAAGCTCATCGCCCTCGTTCCCACCATGGATCCCGAAGCCGAATTC 3'
                                       Li5 primer (reverse complement)
            R  S  M  R  S  Q  G  K  L  I  A  L  V  P  T  M
```

Figure 5

```
  5'---RBS---TSE---ATGGCACCAATGGTGATATCTGAT-3'
                   ||||||||||||||||||||||||
GAATTCGGCACGAGCTCCAATGGCACCAATGGTGATATCTGATAAGGACGAGATGCGGAAATGGTCAAGGTCCATGCGAT
                    M  A  P  M  V  I  S  D  K  D  E  M  R  K  W  S  R  S  M  R  S

CCCAAGGCAAGCTCATCGCCCTCGTTCCCACCATGGGCTTCCTTCACGAAGGCCACCTTTCTCTCGTCAGAGACGCTCAC
  Q  G  K  L  I  A  L  V  P  T  M  G  F  L  H  E  G  H  L  S  L  V  R  D  A  H

AACCACGCTGACCTCGTCGCCGTCTCAATCTATGTCAACCCTGGCCAGTTTTCCCCGACCGAGGACCTTTCCGCATACCC
N  H  A  D  L  V  A  V  S  I  Y  V  N  P  G  Q  F  S  P  T  E  D  L  S  A  Y  P

TTCTGATTTTCAAGGTGATCTCCAAAAACTCATGTCTGTTCCTGGTGGTGTTGATGTTGTTTTCCACCCCCACAATTTGT
 S  D  F  Q  G  D  L  Q  K  L  M  S  V  P  G  G  V  D  V  V  F  H  P  H  N  L  Y

ATGATTACGGTGGTGATGGCGGTGATGCTGTGGCGGAGTGTGGTGGTGATGGGGTGGTGTCTTGTGTTGATAGGAGGAGT
  D  Y  G  G  D  G  G  D  A  V  A  E  C  G  G  D  G  V  V  S  C  V  D  R  R  S

GGTTTTGGGCATGAAACTTGGGTTAGAGCTGAGAAGCTGGAGAAACCCCTTTGTGGGAAGAGTAGGCCTGTTTTCTTTAG
G  F  G  H  E  T  W  V  R  A  E  K  L  E  K  P  L  C  G  K  S  R  P  V  F  F  R

AGGGGTTGCCACCATTGTTACCAAGTTGTTTAATATTGTGGAGCCTGATGTTGCTGTGTTTGGGAAGAAGGACTATCAGC
 G  V  A  T  I  V  T  K  L  F  N  I  V  E  P  D  V  A  V  F  G  K  K  D  Y  Q  Q

AATGGAAAATTATTCAGAGAATGGTTCGAGATCTTGATTTTTCCATAAAAGTGATAGGTTCTGAAGTAATACGTGAGAAA
  W  K  I  I  Q  R  M  V  R  D  L  D  F  S  I  K  V  I  G  S  E  V  I  R  E  K

GATGGCCTAGCAATGAGTTCCCGTAATGTGTACCTATCACCTGAAGAGAGGGAAAAGGCAGTATCTATAAATAAATCATT
```

```
D  G  L  A  M  S  S  R  N  V  Y  L  S  P  E  E  R  E  K  A  V  S  I  N  K  S  L

GTTTAGAGCTAAATCGGCAGCAGAAGATGGACAGATACATTGTGAGAAATTGATAAACTTGGTCGTGCAAAGTATCACCG

 F  R  A  K  S  A  A  E  D  G  Q  I  H  C  E  K  L  I  N  L  V  V  Q  S  I  T  E

AAGCTGGTGGAAGGATTGATTATGCTGAGATTGTTGATCAAAATAATTTGGAGAAAGTGGAATGGATCAAGGGTCCTGTT

  A  G  G  R  I  D  Y  A  E  I  V  D  Q  N  N  L  E  K  V  E  W  I  K  G  P  V

GTCTTCTGTGTTTCTGCATGGTTTGGGAAAGCCAGGCTTATAGACAACATAGAAATCAACTTGTAAATGGAAGTAAGATT
                                                  ||||||||||||||||||||
                                      3'-TTGTATCTTTAGTTGAACATT---5'

V  F  C  V  S  A  W  F  G  K  A  R  L  I  D  N  I  E  I  N  L  *

GATCTAACCTTGTGAATAATCTCAGACATGGACCATATGATTAGTAGTTCTGGCATTTCATGGGGTATAGACTTCATTCT

ACAAGCCATGATATGACTACTTGTAGATGTATTTTACTACCTCATGAAATTCTAGGAGCTGCTTCTATTTGTTGGTGATG

GTATAATATTTTGCAGAGCCACCACTCCAGAGGAAAACAAAATTAGAGAAATCTTGCTTATGTATCAAAGTGCCCCAGGT

TTACTCATTAATCTAGATAAATCTGAGCTTTCTTTAGGCTGATGTACGCCTAGAGATAGACAAACATAATTCTGGTGCTG

GATAAAATTAACGCATTGGATTCCCATTTGAAATAAAAAAAAAAAAAAAAAACTCGAG
```

Figure 6: Anion exchange chromatography of recombinant *Lotus japonicus* pantothenate synthetase

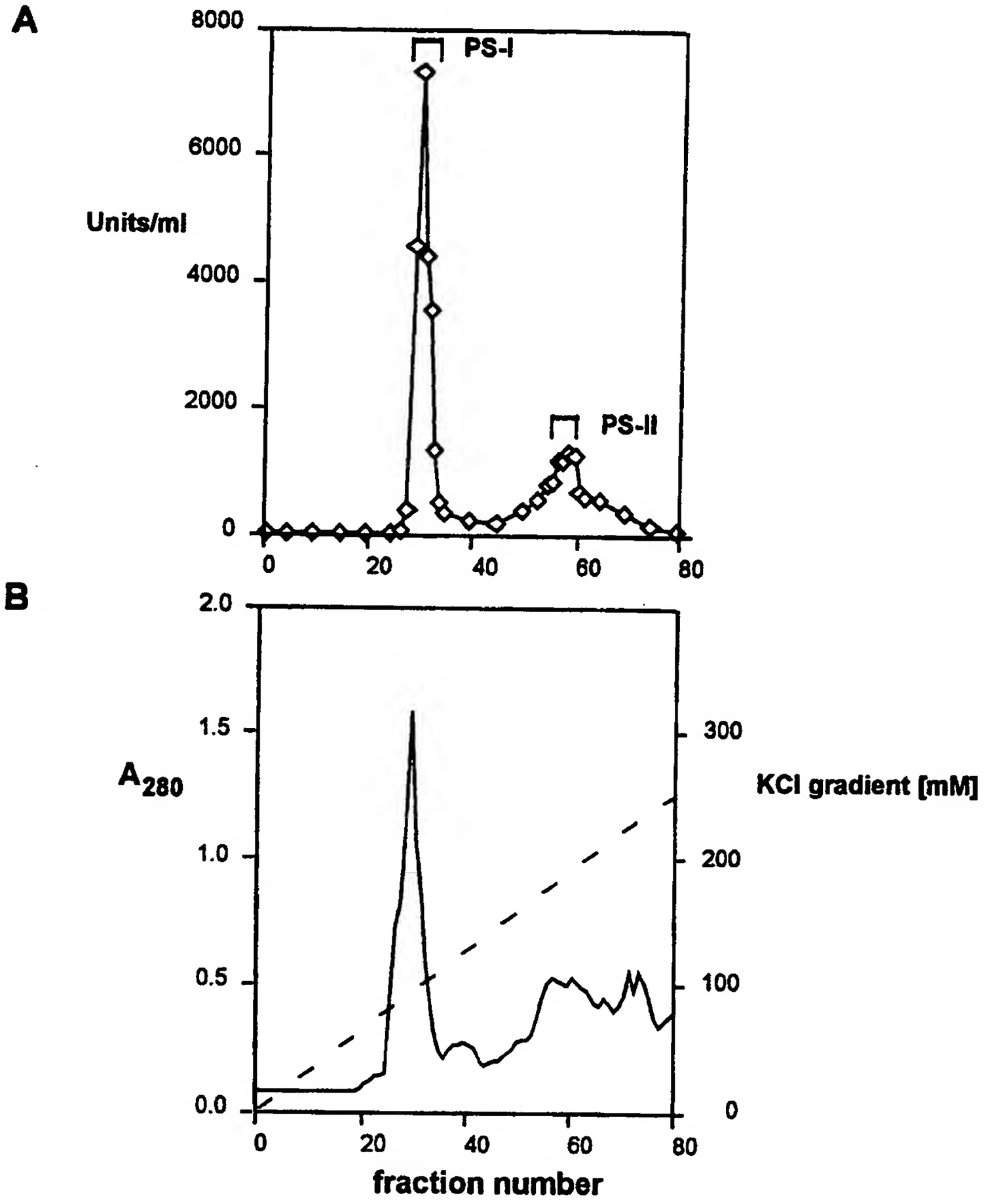
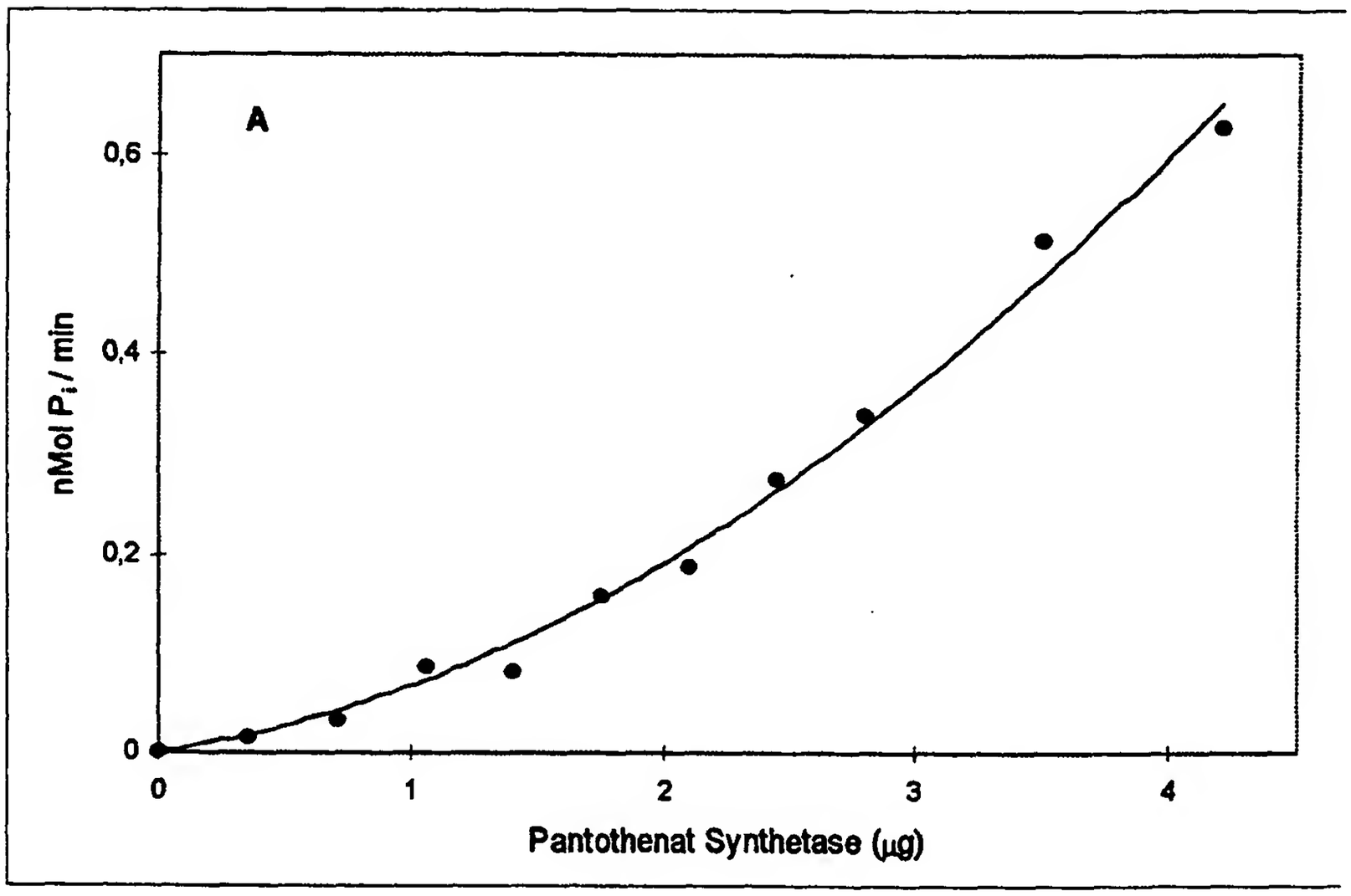
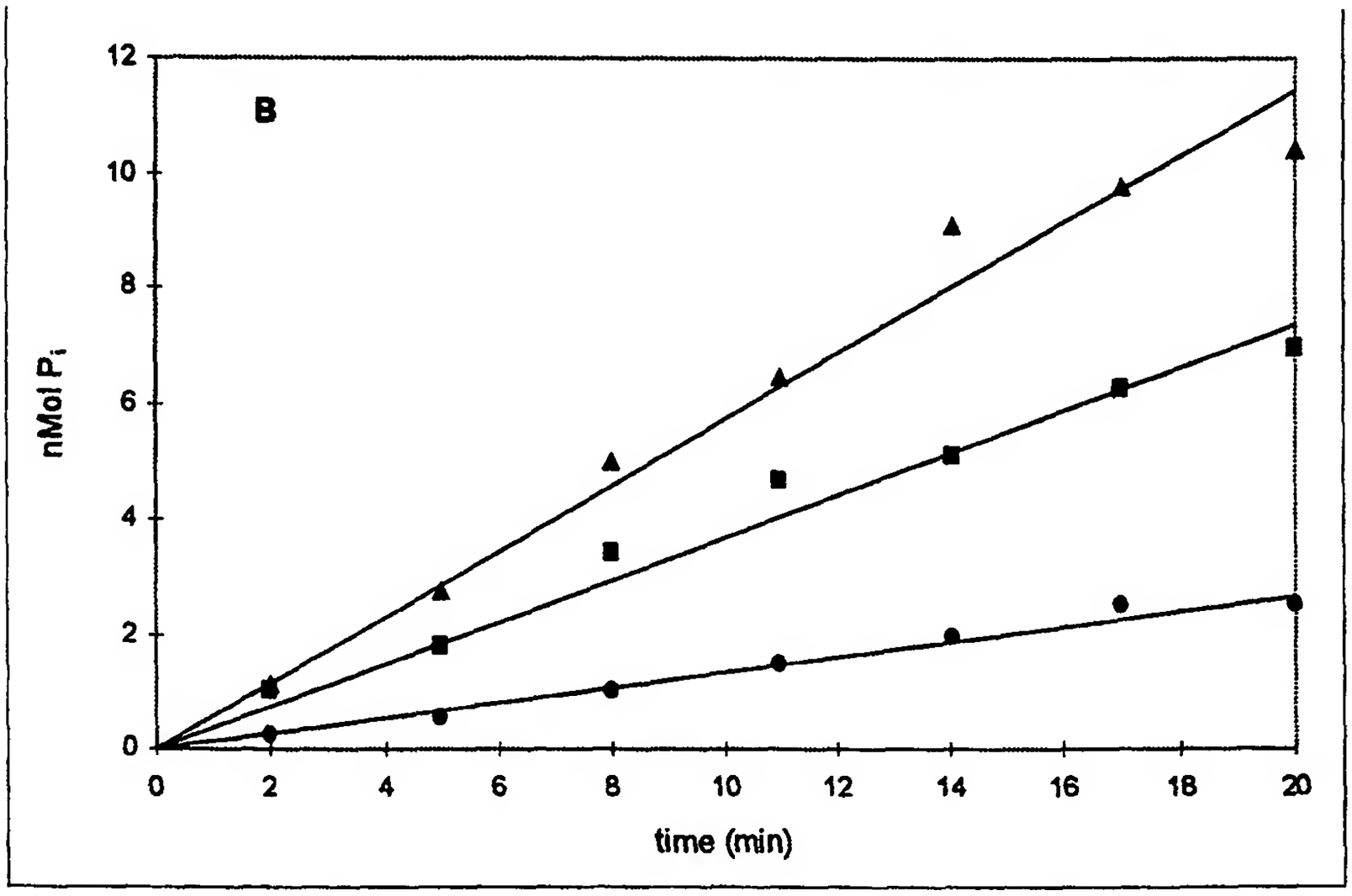


Figure 7: High-throughput assay for recombinant *Lotus japonicus* pantothenate synthetase.

Protein dependence and time course of the pantothenat synthase assay.





INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 98/03261

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/00 C12N15/52 C12N15/29 C12N15/82 C12N15/62
C12N15/11 C12Q1/68 C12Q1/25 G01N33/50 A01N61/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	U. GENSCHEL ET AL., : "Comparison of the biosynthetic pathways leading to pantothenate (vitamin B-5) in bacteria and higher plants" JOURNAL OF EXPERIMENTAL BOTANY, vol. 46, no. suppl., 1995, page p17 XP002092389	1
Y	see abstract P2.44	5-7,13, 20-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 1999	23/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 98/03261

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	C.E. JONES ET AL., : "Cloning and sequencing of the Escherichia coli panB gene, which encodes ketopantoate hydroxymethyltransferase, and overproduction of the enzyme." JOURNAL OF BACTERIOLOGY, vol. 175, 1993, pages 2125-2130, XP002092390 see page 2125, left-hand column; figures 1,3 ---	5-7,13, 20-22
A	WO 96 01326 A (AGREVO UK LTD) 18 January 1996 see the whole document ---	1-22
A	DATABASE WPI Week 9715Derwent Publications Ltd., London GB; AN 97-163337 RD393067 : "Large-scale screening of potentially herbicidal compounds- which act by inhibiting the enzyme 4-hydroxy-phenyl pyruvate di-oxygenase" , 10 January 1997 XP002092397 see abstract	1-22
P,A	-& WO 98 04685 A (AMERICAN CYANAMID COMPANY) 5 February 1998 see the whole document ---	1-22
A	C.E. JONES ET AL., : "Evidence for the pathway to pantothenate in plants" CANADIAN JOURNAL OF CHEMISTRY, vol. 72, no. 1, 1994, pages 261-263, XP002092391 see the whole document ---	1
A	P.A. LANZETTA ET AL., : "An improved assay for nanomole amounts of inorganic phosphate" ANALYTICAL BIOCHEMISTRY, vol. 100, no. 1, 1979, pages 95-97, XP002092392 cited in the application see the whole document ---	1,14,15

-/--

3

INTERNATIONAL SEARCH REPORT

Int' 'onal Application No
PCT/EP 98/03261

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	S. CHIFFLET ET AL., : "A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases" ANALYTICAL BIOCHEMISTRY, vol. 168, no. 1, 1988, pages 1-4, XP002092393 cited in the application see abstract	1,14,15
A	K. MIYAKE ET AL., : "Enzymological properties of pantothenate synthetase EC-6.3.2.1 from Escherichia coli B" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, vol. 24, no. 3, 1978, pages 243-253, XP002092394 see the whole document	1,6,7,9, 17
A	J.E. CRONAN ET AL., : "Genetic and biochemical anlysis of pantothenate biosynthesis in Escherichia coli and Salmonella typhimurium" JOURNAL OF BACTERIOLOGY, vol. 149, no. 3, 1982, pages 916-922, XP002092395 cited in the application see abstract	1,6,7,9
P,X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, - 1 August 1997 XP002092396 HINXTON, GB AC= Y10253. Oryza sativa panC gene; pantoate-beta-alanine-ligase. see abstract	1,5-7,9
P,X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, - 1 August 1997 XP002092569 HINXTON, GB AC= Y10252. Lotus japonicus panC gene; pantoate-beta-alanine-ligase. see abstract	1-4,9

INTERNATIONAL SEARCH REPORT

.national application No.
PCT/EP 98/03261

B x I Obs rvations where certain claims wer found unsearchable (C ntinuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

SEE ADDITIONAL SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ EP 98/03261

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1, 5-7, 13-22 and partially 8-12

An isolated DNA molecule encoding a protein from a plant, wherein the plant is Oryza sativa and which protein has pantothenate synthetase activity.

2. Claims: 2-4 and partially 8-12

An isolated DNA molecule encoding a protein from a plant, wherein the plant is Lotus japonicus and which protein has pantothenate synthetase activity.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int 'onal Application No
PC1/EP 98/03261

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9601326 A	18-01-1996	AU 2923295 A	25-01-1996
		EP 0769067 A	23-04-1997
		JP 10502250 T	03-03-1998
		US 5786165 A	28-07-1998